Deanship of Graduate Studies

Al-Quds University

Phytochemical screening of *Cuscuta palaestina, Gundelia tournefortii, Pimpinella anisum* and *Ephedra alata* natural plants and their *in-vitro* cytotoxic effects

Sadam Ahmad Mahmoud Makhamra

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Jerusalem–Palestine

1437/2016

Declaration

I certify that this thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and this thesis has not been submitted for the higher degree to any other university or institute.

Signed: ……..

Sadam Ahmad Mahmoud Makhamra

Date: 3-8-2016

Abstract

The aim of this research is to assess the *in-vitro* antiproliferative activities of the crude extract, preparative HPLC fractions and pure active compounds in selected Palestinian plants, namely *Cuscuta palaestina, Gundelia tournefortii, Pimpinella anisum and Ephedra alata.* Chromatographic and spectroscopic techniques such as analytical HPLC-PDA, preparative HPLC-PDA, GC-MS, ^{1}H-NMR, and LC-MS and biological assays were used to achieve this goal.

Cuscuta palaestina is listed as native plant of flora palaestina. The methanolic and hexane extracts were used to determine its activity against human colorectal carcinoma cell line. The *in-vitro* results using MTT and the LDH leakage assays, gave EC_{50} values of 71.55 ± 4.75 µg/ml for methanol extract and 175.21 ± 3.89 µg/ml for hexane extract. The methanolic extract exerted a significant anti-proliferative effect on the HCT-116 cancer cell line. Sesamin and two other Phytosterols namely Campesterol and Stigmasterol, have been identified in this plant and therefore could be the major source for their anticancer activity. The Sesamin percentage in the methanolic extract is found to be three times more than Sesame seeds.

Gundelia tournefortii is an Artichoke-like edible wild thistle is commonly used in Palestinian Cuisine. Its anticancer effects on the Human colon carcinoma cell line was investigated. Methanol and hexane extracts were found to exert considerable antitumor activity against HCT-116 cancer cell line with EC_{50} values of 303.3 ± 12 µg/ml, 313.3 ± 18.6 µg/ml respectively, while the aqueous extract was inactive. Phytochemical identities in both methanol and hexane extracts using GC-MS revealed about seventy compounds. Six potential compounds are β-Sitosterol, Stigmasterol, Lupeol, Gitoxigenin, α-Amyrin and Artemisinin. Eight fractions were collected by preparative-HPLC and tested *in-vitro*. The cytotoxic effect of fraction VIII was found to be the highest against HCT-116 cancer cell line.

Pimpinella anisum L. (Anise) was found to have potential anticancer activity on human prostate cancer cell line (PC-3). Six fractions of anise methanolic extract were collected using preparative-HPLC. The obtained EC_{50} value as measured for fractions II, III, IV, and V by the MTT assay was 0.5 ± 0.10 mg/ml, 0.3 ± 0.11 mg/ml, 0.49 ± 0.12 mg/ml, 0.23 ± 0.10 mg/ml, respectively.

Ephedra alata acquired recently a tremendous reputation after the use of its stems to heal a Palestinian farmer from Jenin. The *in vitro* antiprolliferative activity of its methanolic extract gave EC_{50} value of 25 ± 1.2 µg/ml by using MTT assay against HepG2 cell line (human liver cancer).

Therefore, the four investigated Palestinian plants could be further investigated to reach new anticancer lead or drug and to study mechanism of action and evaluate efficiency and toxicity for *in vivo* models.

Keywords: Cytotoxicity, *Cuscuta palaestina*, antiproliferation, anticancer, HCT-116 (human colon cancer cell line); MTT, LDH, EC_{50}, GC-MS analysis. *Gundelia tournefortii*, phytochemicals, *Pimpinella Anisum*, Anise PC-3 (human Prostate cancer cell line); *Ephedra alata* HepG2 (human liver cancer cell line);

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My deepest respect and thanks to Prof. Dr. Anwar Rayan, head of drug discovery informatics laboratory, QRC-Qasemi Research Center for his collaboration in the test of anticancer activities of plants fractions.

Finally, special thanks is extended for my parents for encouraging me to seek knowledge and for their help and continuous support.

Dedication

This thesis is dedicated to:

The sake of Allah, my Creator and my Master,

The teacher of teachers, Prophet of humanity Mohammad -peace be upon him,

My beloved parents, who never stop giving of themselves in countless ways, and for endless support, and encouragements.

Table of Contents

List of Abbreviations and symbol

Abbreviations	Theme
WHO	World Health Organization
HPLC	High-Performance Liquid Chromatography
PDA	Photodiode array
GC-MS	Gas Chromatography - Mass Spectrometry
ATCC	American Type Culture Collection
LOD	Limit of Detection
LOQ	Limit of Quantitation
NIST	National Institute of Standards and Technology
m/z	Mass-to-Charge ratio
^{1}H-NMR	Proton Nuclear Magnetic Resonance
HR-ESi-MS	High Resolution-Electrospray Ionization-Mass Spectrometry
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
2D-NMR	Two Dimensional Nuclear Magnetic Resonance
COSY	Two-dimensional ^{1}H-^{1}H correlation spectrometry
HMQC	Heteronuclear Multiple-Quantum Correlation spectroscopy
MTT	Micro-culture tetrazolium
LDH	Lactate dehydrogenase
ELSD	Evaporative light scattering detector
Fig.	Figure
µ	Micro
EC_{50}	Half maximal effective concentration
IC_{50}	Half maximal inhibitory concentration
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
IU	International unit
U	Unit
ml	Milliliter
LC-QTOF	Liquid Chromatography - Quadrupole Time-of-Flight
Da	Dalton (Molecular weight unit)
PUBMED	A bibliographic database, comprises more than 26 million citations for biomedical literature from MEDLINE, life science journals, and online books. Citations may include links to full-text content from PubMed Central and publisher web sites.

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Chapter One

Introduction

1. Introduction

1.1. Cancer diseases and their therapy

Cancer is the second leading cause of death in the world after cardiovascular disease. Every year, more than 8.2 million cancer deaths are reported worldwide. The 14.1 million new cases each year, were 32.5 million persons are alive with cancer. It is about 68% occurring in the developing countries alone. WHO predicts 23.6 million cases in the year 2030s [1,2].

Cancer is uncontrolled dividing and growth of the abnormal cell due to their internal factors (inherited genetic mutations, hormones, immune conditions), and external factors (unhealthy diet, chemicals, radiation, viruses, lifestyle), and environmental change (smoking, air pollution, sunlight, work place like UV radiation). Cancer cells, which are already irreversibly developed, have the capability to escape apoptosis by a number of ways that involve transformation, dysregulation of apoptosis, proliferation, invasion, angiogenesis and metastasis. Extensive research during the last 35 years has revealed much about the biology of cancer. While the normal body cells grow, dividing, and die in an orderly fashion. Mutations in certain genes, mainly in proto-oncogenes, and tumor-suppressor genes, can contribute to the development of cancer [3].

There are many types of cancers infect humans like lung, breast, prostate, ovarian, bladder, neck, blood, colorectal, stomach, esophageal, pancreatic, liver, brain cancer, and other neoplasm. Cancer is a complex disease involving numerous temporospatial changes in cell physiology, which ultimately lead to malignant tumors. Abnormal cell growth (neoplasia) is the biological endpoint of the disease. Tumor cell invasion of surrounding tissues and distant organs is the primary cause of morbidity and mortality for most cancer patients. The biological process by which normal cells are transformed into malignant cancer cells has been the subject of a large research effort in the biomedical sciences for many decades. Despite this research effort, cures or long term management strategies for metastatic cancer are as challenging today as they were 44 years ago when President Richard Nixon declared a war on cancer [4,5].

When cancer spreads to other parts of the body, this is called metastasis. Metastases can occur when cancer cells enter the blood stream or lymph system, these systems

circulate all over the body and allow the cells to travel to another part of the body, which increases the seriousness of the disease [6].

There are four main types of cancer:

1) Carcinomas – cancers of the organs.
2) Sarcomas – cancers of the muscles, bone, cartilage, and connective tissue.
3) Lymphomas – cancers of the lymphatic system.
4) Leukemia's – cancers of the blood-making system.

Fig. 1 shows the estimated cancer mortality worldwide in 2012 [7].

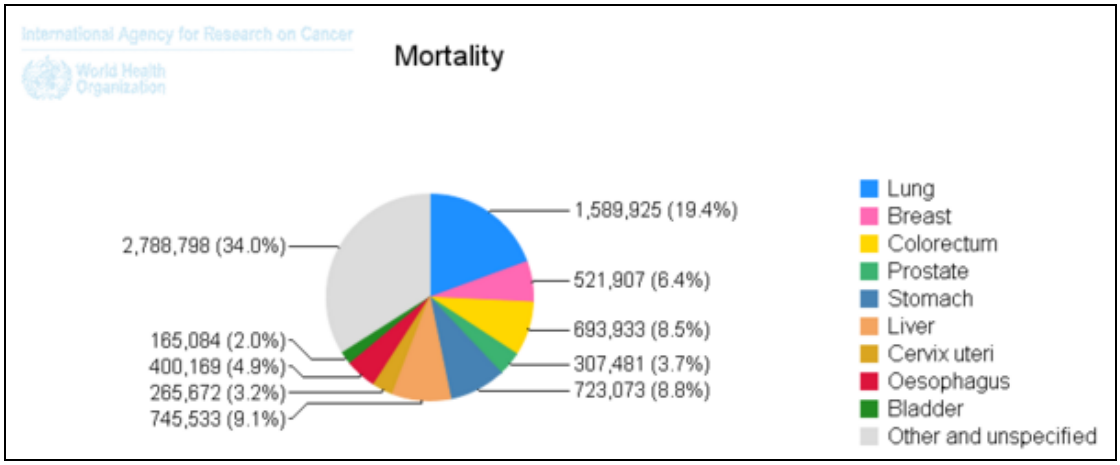
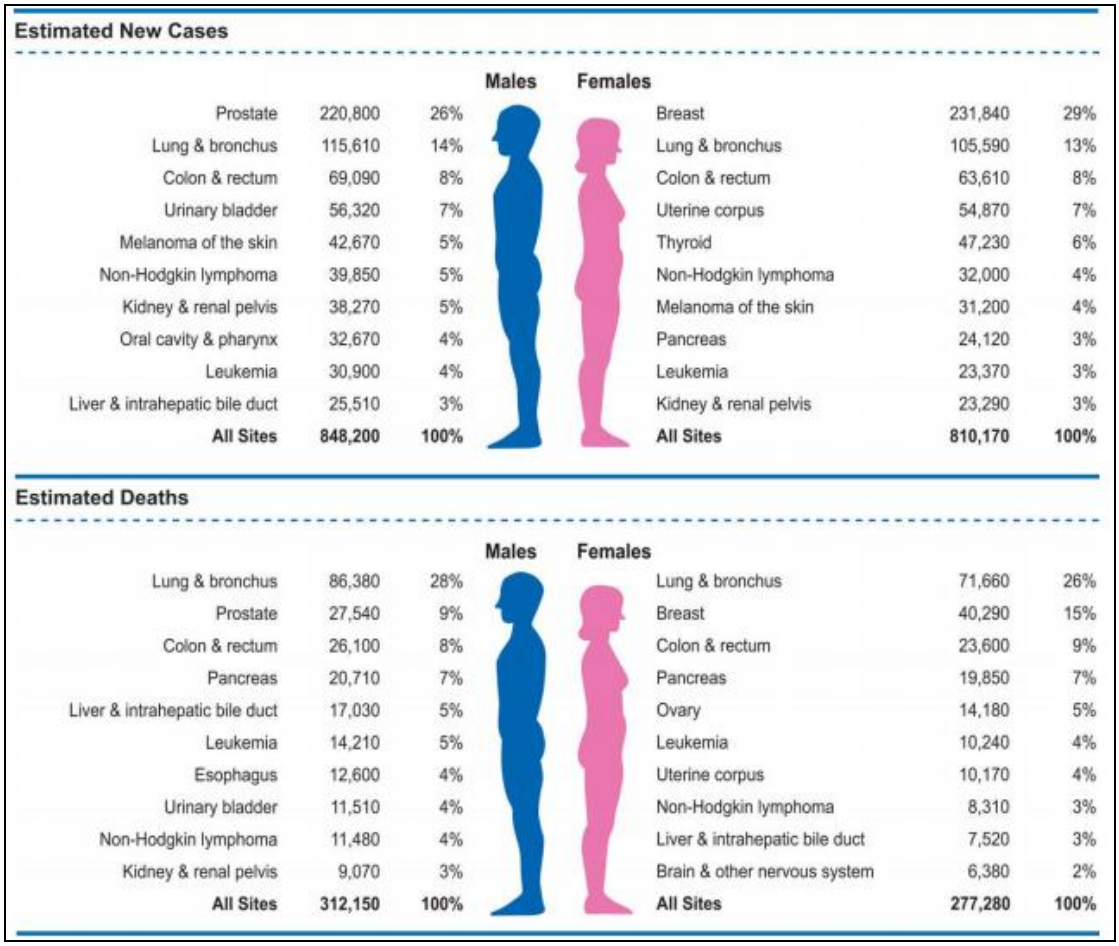


Fig. 1: Estimated cancer mortality for both sexes worldwide in 2012.

Each year, the American Cancer Society for example estimates the numbers of new cancer cases and deaths that will occur in the United States in the current year and compiles the most recent data on cancer incidence, mortality, and survival. Fig. 2 shows the leading 10 types of cancer estimates in the US at 2015 [8].



Estimated New Cases

Males			Females		
Prostate	220,800	26%	Breast	231,840	29%
Lung & bronchus	115,610	14%	Lung & bronchus	105,590	13%
Colon & rectum	69,090	8%	Colon & rectum	63,610	8%
Urinary bladder	56,320	7%	Uterine corpus	54,870	7%
Melanoma of the skin	42,670	5%	Thyroid	47,230	6%
Non-Hodgkin lymphoma	39,850	5%	Non-Hodgkin lymphoma	32,000	4%
Kidney & renal pelvis	38,270	5%	Melanoma of the skin	31,200	4%
Oral cavity & pharynx	32,670	4%	Pancreas	24,120	3%
Leukemia	30,900	4%	Leukemia	23,370	3%
Liver & intrahepatic bile duct	25,510	3%	Kidney & renal pelvis	23,290	3%
All Sites	**848,200**	**100%**	**All Sites**	**810,170**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	86,380	28%	Lung & bronchus	71,660	26%
Prostate	27,540	9%	Breast	40,290	15%
Colon & rectum	26,100	8%	Colon & rectum	23,600	9%
Pancreas	20,710	7%	Pancreas	19,850	7%
Liver & intrahepatic bile duct	17,030	5%	Ovary	14,180	5%
Leukemia	14,210	5%	Leukemia	10,240	4%
Esophagus	12,600	4%	Uterine corpus	10,170	4%
Urinary bladder	11,510	4%	Non-Hodgkin lymphoma	8,310	3%
Non-Hodgkin lymphoma	11,480	4%	Liver & intrahepatic bile duct	7,520	3%
Kidney & renal pelvis	9,070	3%	Brain & other nervous system	6,380	2%
All Sites	**312,150**	**100%**	**All Sites**	**277,280**	**100%**

Fig. 2: Ten leading cancer types for the estimated new cancer cases and deaths by sex, United States, 2015.

Depending on the type of cancer, age of patient, sex of patient (male or female), tumor location, and stage, there are many option of treatment of cancer, which include Surgery, Radiation therapy, Chemotherapy, Immunotherapy, Hormone therapy, targeted therapy, and also extracts or leads from herbal medicine.

1.2. Secondary metabolites from natural product

In the simplest terms, natural product is a molecule that is produced from plants, animal, microbes, micro-organism [9]. The primary metabolites, in contrast, such as acyl lipids, nucleotides, amino acids, and organic acids, are found in all plants and perform metabolic roles that are essential and usually evident [10]. While the secondary metabolite facilitates the primary metabolism in plants contain a huge amount of compounds that are not strictly necessary for growth and development, but which play a crucial role in defense mechanism and adaption to the environment,

which include terpenes, phytosterols, steroids, phenolics and alkaloids, exhibit a wide range of biological activities and have immense potential application in the chemical-pharmaceutical industries as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives [11]. Phytochemicals are the active secondary metabolite components, present in a plant that account for its medicinal properties. As a central theme of exploration bordering chemistry and biology, natural products research focuses on the chemical properties, structures, chromatographic isolation, biosynthesis and biological functions of active secondary metabolites. Lignans for example are large class of secondary metabolites in plants, with numerous biological effect in mammals, including antitumor and antioxidant activities. Sesamin specifically is the most abundant furofuran-class lignan in sesame seeds. Recently, sesame cytochrome P450, CYP81Q1, as the first P450 enzyme responsible for lignan biosynthesis has been identified [12]. Pinoresinol is synthesized by the stereospecific coupling of two units of achiral *E* -coniferyl alcohol in the presence of a dirigent protein (DIR). CYP81Q1 converts pinoresinol to sesamin via piperitol, and has been designated piperitol/sesamin synthase (PPS) as shown in Fig. 3.

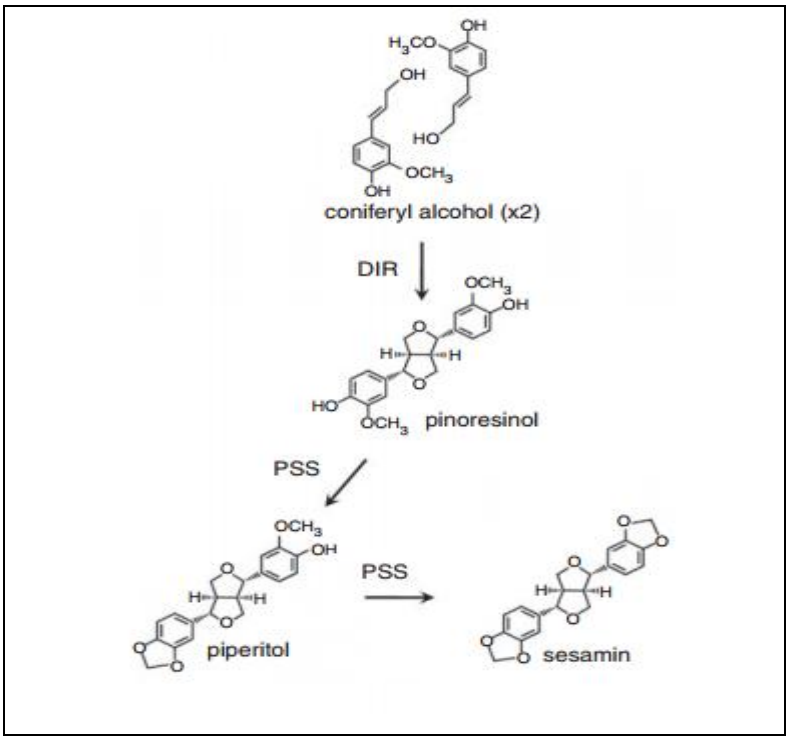


Fig. 3: Lignan biosynthesis pathways for sesamin. DIR, dirigent protein; PSS, piperitol/sesamin synthase.

The major sterol is typically sitosterol, followed by campesterol, stigmasterol, and various minor sterols. Sitosterol and stigmasterol play crucial roles in cellular membranes, especially in the plasma membrane. Campesterol is the precursor to the brassinosteroids (BRs), the only plant steroid hormones identified thus far. Signaling roles for additional plant steroid compounds are not known thus far (Fig. 4).

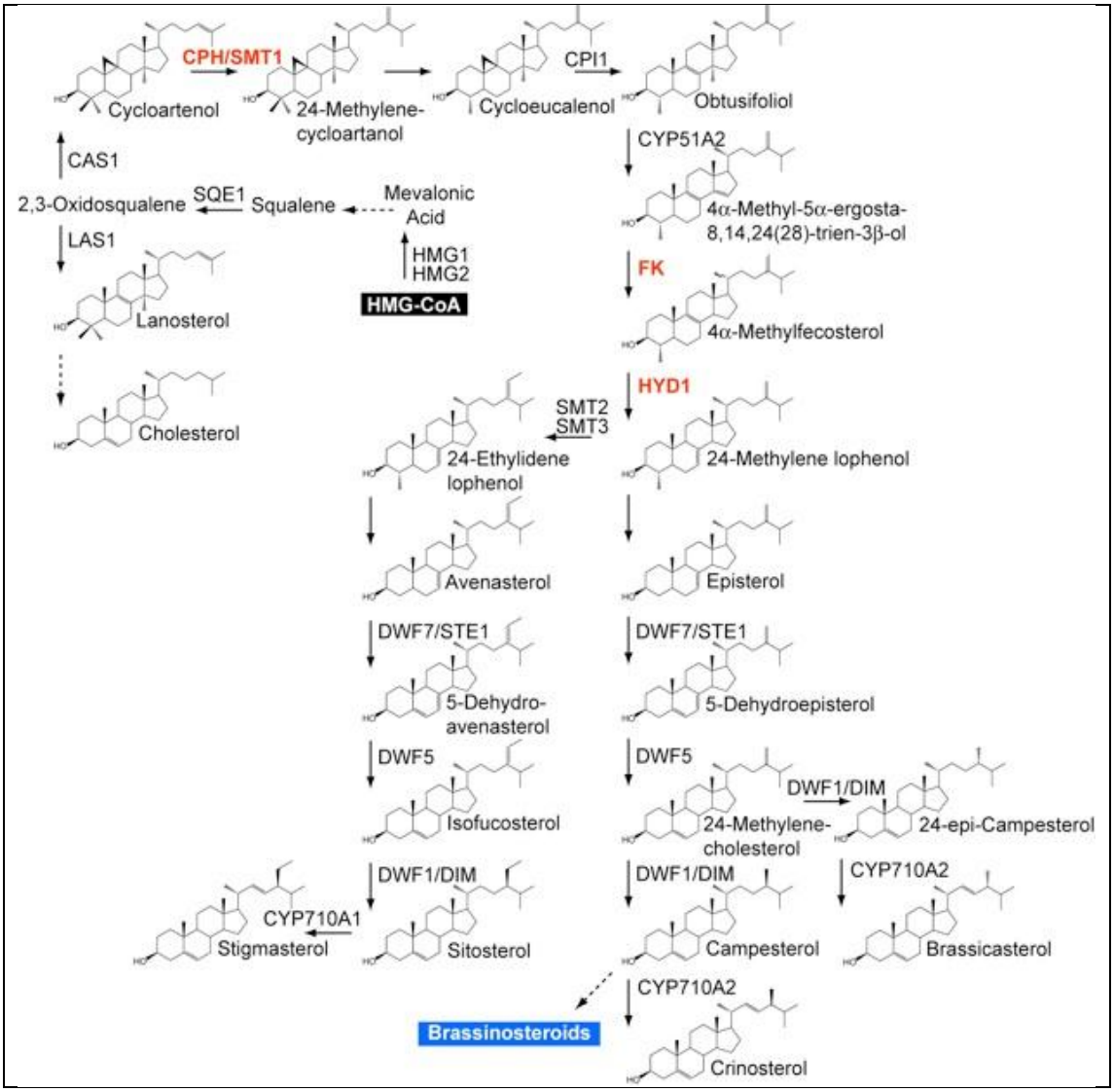


Fig. 4: Biosynthesis of important phytosterols

1.3. Medicinal plants and traditional medicine

Throughout history, man used various natural materials as a remedy for various diseases. In the past few decades, most natural products were replaced with synthetic drugs that were based on modern chemistry and biotechnology between 1990s and 1997 in the U.S.A. However, we are recently witnessing a vastly growing and renewed interest in natural medicines in western countries. In particular, the herbal medicine market has distributed and became prosperous in pharmacies and many stores [13]. Medicinal plants are commercially available, cheaper, as compared to modern drugs. Thus, the various combinations of the active components of these plants after chromatographic isolation and spectroscopic identification can be made and may be further assessed for their synergistic effects. The bioactive compounds have a tangible effect on the living organism, tissue or cell [14].

Recent years have witnessed a renewed interest in plants as an alternative avenue to discover new pharmaceuticals during 1990s to now. This interest is driven by both academia and pharmaceutical industry and has led to the espousal of crude extracts of plants for self-medication by the general public. Plants used in traditional medicine; therefore, have an important role to play in the maintenance of health worldwide, and in the introduction of new treatments [15,16].

More than 60 percent of the currently used anticancer agent are derived in one way or another from natural sources, including plants [17,18]. WHO estimates that up to 80 percent of the world's population relies on herbal medicine for some aspect of primary health care [19].

Herbal medicine has been universally accepted and has had a great impact on the worlds of health and international trade [20]. However, screening medicinal plants in *in vitro* systems is the first step in finding potential anticancer compounds [21]. Where estimated number of plant species on earth ranges between 500,000 and 1,250,000 and less than 10 percent of these have been studied chemically and pharmacologically for their potential medicinal value [22].

In Palestine, traditional remedies are usually part of its culture and religious life. , Still however few herbal products used in folk medicine were investigated and recorded.It has a rich and prestigious heritage of herbal medicines; in addition more than 700 species of medicinal plant are known to exist, and approximately 63 of these are activity used for the preparation of traditional medicines [23]. Medicinal plants in

Palestine as well as in other developing countries has high pharmaceutical and ecological value, and have important elements of indigenous medical systems [24,25].

1.4. Natural products used as anticancer agent, isolation and identification

The terrestrial plants remain important source of new chemical entity to treatment of cancer in clinical trials. There are many classes of plant-derived cytotoxic natural products against cancer cells, and studied for further improvement and development of anticancer agent. For example, Vincristine, irinotecan, etoposide, paclitaxel, Viniblastine, and Camptothecin. Since the oceans cover more than 70% of the earth surface, and the marine environment is highly diverse, marine organisms would be also a wonderful source of biologically active anticancer molecules. For example, aplidine, didemin, dolastatin, citarabine, homophymines, jaspamide, and from micro-organisms like, dactinomycin, bleomycin and doxorubicin. Examples of the biomolecules responsible for the anticancer activity microbial sources are such as dactinomycin, bleomycin, and doxorubicin [26,27,28].

Besides, this there is numerous agents from fruits and vegetables that can be used in anticancer therapy. The agents include curcumin (turmeric), resveratrol (red grapes, peanuts and berries), genistein (soybean), diallyl sulfide (allium), S-allyl cysteine (allium), allicin (garlic), lycopene (tomato), capsaicin (red chilli), diosgenin (fenugreek), 6-gingerol (ginger), ellagic acid (pomegranate), ursolic acid (apple, pears, prunes), silymarin (milk thistle), anethol (anise, camphor, and fennel), catechins (green tea), eugenol (cloves), indole-3-carbinol (cruciferous vegetables), limonene (citrus fruits), beta carotene (carrots) [29].

Isolation of secondary metabolite from higher plants (leaves, flowers, stems, seeds or fruits) are examples of natural bioactive compounds from potential sources as flavonoids and essential oil, and testing for biological activity has to be done after a purification process. The active agent can be isolated depends on the, stability, and quantity of extract in the plant natural product in order to fully elucidate the structure of the active compounds [30]. The majority of isolation procedures still utilize simple extraction procedures with organic solvents of different polarity using infusion or by sonication [31,32]. Other methods include Soxhlet extraction which is a popular method due to its reduced solvent consumption; however, thermo-labile compounds

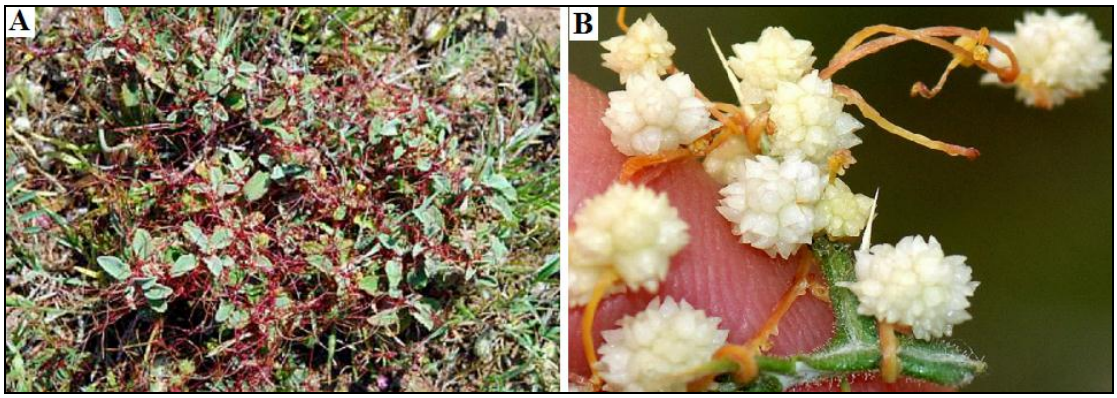
might be degraded during the extraction process. For liquid samples, extraction by organic solvents or heterogeneous solvent mixtures can be used, either simply in a separating funnel or similar to a Soxhlet apparatus in a perforator [33]. Chromatographic techniques utilized gas chromatography (GC) and High-Performance Liquid Chromatography (HPLC). Hyphenation of HPLC with different spectroscopic detectors like Mass Spectrometry (MS), Photodiode array (PDA) and Evaporative Light Scattering Detector (ELSD) are available techniques. Proton Nuclear Magnetic Resonance (^{1}H-NMR), Carbon-13 Nuclear Magnetic Resonance (^{13}C-NMR) and 2D-NMR spectroscopy offers ways to identification of plant material. Preparative HPLC is an excellent technique used for fractionation and isolation of pure active compounds with high peak resolution power and selectivity. In case of analyzing biological material containing volatile constituents like essential oils, GC-MS analysis still represents the method of choice, taking advantage of the unsurpassed peak capacity of capillary GC columns. Electron impact mode is usually used and there are libraries such as NIST which contains about half million compounds for matching the compounds identity. Headspace solid-phase micro-extraction (SPME) or steam distillation (SD) extraction methods can be used to collect the volatile fractions from small amounts of plant material [34].

1.5. Screening anticancer bioactivity from four Palestinian plants

1.5.1. *Cuscuta palaestina* plant

The first plant that we will extract it and scan its anticancer activity is called *Cuscuta palaestina.* It is a plant that belongs to *Cuscuta* species, and Convolvulaceae family. It is an extensive climber parasitic plant that more often called “dodder” in English and *Halook* or *Hamool Falastini* (الحامول الفلسطيني) in native Arabic (see pictures A, and B).

The mature plants have no connection to the ground, so it parasites on the host plants. The plant has no chlorophyll and cannot make its own nutrients by photosynthesis. The stem consists of thread filaments that grow and attach themselves to nearby host plants. *Cuscuta,* is one of the largest and most economically important lineages of parasitic plants. The genus has a sub cosmopolitan distribution which is more than 75% of the species diversifying in the world, the *Cuscuta* plant included a genus of 200 species of stem parasites [35].



Pictures A and B: Climbing *Cuscuta Palaestina* parasite, stem and flowers respectively.

Cuscuta palaestina is considered one of the most dangerous parasitic plants, causing huge losses of various agricultural crops, due to penetrate of leaves and stem of wild host plant. Botanical description of *Cuscuta palaestina*, is leafless plant are widespread in the east Mediterranean region like Turkey, Cyprus, Lebanon, Palestine, Egypt, Syria, Jordan, Iraq and Iran [36]. It have a flowers 1.5-2 mm, sessile in very small, dense head 4-6 mm across; corolla lobes with erect hooded tips. Capsule round seeds about 1 mm [37]. The classification of *Cuscuta palaestina* is shown in the table 1.

Table 1: Classification of *Cuscuta palaestina*.

Kingdom	**Plantae**
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Solanales
Family	Convolvulaceae
Genus	*Cuscuta*
Species	*Cuscuta palaestina*.

Colorectal cancer develops in the colon or the rectum, also known as the large intestine is the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the US. The American Cancer Society estimates that 136,830 people will be diagnosed with colorectal cancer and 50,310

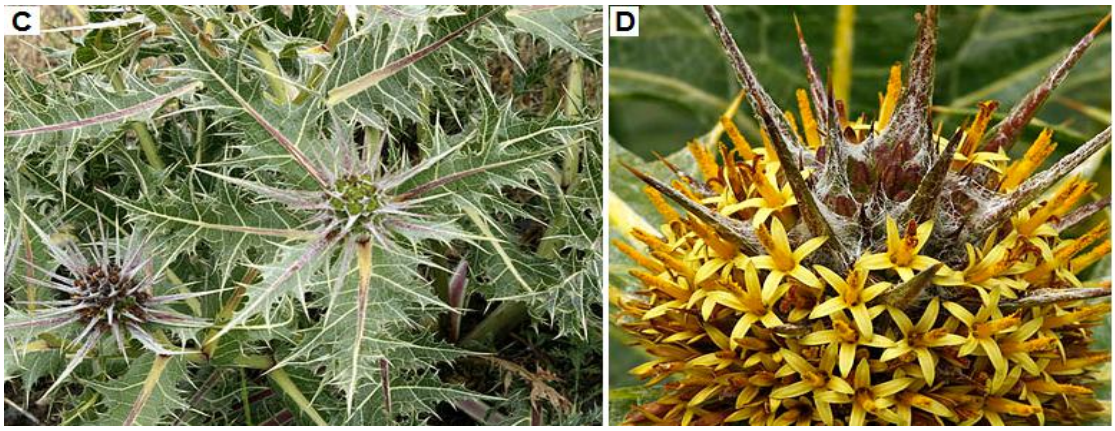
people will die from the disease in 2014 [38]. As part of our efforts to find potential sources of natural agents that inhibit cancer development, we have investigated the anticancer effects of *Cuscuta palaestina* crude extract on the colon carcinoma cell line.

According to intensive literature survey, there is no GC-MS method yet reported on the simultaneous determination of the phytochemicals present in *Cuscuta palaestina*. The main constituents of the methanol and hexane extracts were explored and investigated for the first time by using GC-MS in the electron impact mode. HPLC-PDA was used to quantitative the active nonvolatile compounds present in *Cuscuta palaestina* extracts by different solvents.

Since parasitic and hemiparasitic plants are endangered by herbivores and microorganisms, they protect themselves by relying on the natural chemical defense of the respective host plant, or by mechanical protection (thorns, spines, or trichomes).

1.5.2. *Gundelia tournefortii* plant

The second plant is *Gundelia tournefortii* L. (Akoub in English, العكوب الجبلي in Arabic) is a spiny perennial flowering plant from Asteraceae (Compositae) family. It have rosette leaves, and have purple to yellow flower in spring and early summer (see pictures C and D). *Gundelia tournefortii* thistle is a vegetable, just like artichoke that grows in the semi-desert area of many countries in the Mediterranean region Particularly in Palestine Iran, Jordan, Syria, Azerbaijan, Armenia, Anatolia and other countries.



Pictures C and D: Spiny plant of *Gundelia tournefortii*, and its flowers respectively.

The classification of *Gundelia tournefortii* is shown in table 2. *G. tournefortii* is an important food source and a well-known medicinal plant in Eastern Anatolia. The therapeutic effects of medicinal plants are known to be closely related to their antioxidant capacities from seed. Traditionally, *G. tournefortii* is used for treatment of hepatoprotective (liver diseases), diabetes, chest pain, heart stroke, gastric pain (stomach), vitiligo, diarrhea and bronchitis, epilepsy. It is also reported to have hypoglycemic, laxative, sedative, anti-bacterial, anti-inflammatory, anti-parasite, and antiseptic [39]. Nature is the best cook factory given the fact that natural products have been optimized to interact with the biological systems through a long natural selection process [40].

The traditional recipes to make the akoub are different according to different localities. It is mainly prepared by taking the young stems with the head after taking off the prickles by a scissor and frying them in olive oil as an omelet, or by adding it to meat chops until they are well done. *G. tournefortii* is still collected wild by local Palestinian populations and considered of high market potential because of its health benefits reputation [41].

Table 2: Scientific classification of *Gundelia tournefortii*.

Kingdom	**Plantae**
Subkingdom	*Tracheobionta*-Vascular plants
Superdivision	*Supermatophyta*-Seed plants
Division	*Magnoliophyta*-Flowering plants
Class	*Magnoliopsida*-Dicotyledons
Subclass	*Asteridae*
Order	*Asterales*
Family	*Asteraceae*-Aster
Tribe	*Gundelieae*
Genus	*Gundelia*
Species	*tournefortii* L.

The GC-MS was utilized to explore the potential phytochemicals responsible for the anticancer activity. The extracts of this plant was tested for their anticancer activity

(human colon cancer cell line). This is the first scientific study to report about the potential benefits of consuming wild edible *Gundelia tournefortii* on cancer and it's phytochemical contents.

1.5.3. *Pimpinella anisum* plant

The third plant that is very famous to all Palestinians is the *Pimpinella anisum* L. (Anise in English, اليانسون in Arabic). It belongs to Apiaceae (Umbelliferae) family, which consists of 300-455 genera and 3000-3750 species globally. Anise is an annual herb growing to 30-50 cm with white flower and small green to yellow seed as shown in pictures E and F. Anise seed is important natural raw material, which is used for pharmaceutics, perfumery, food and drink. It grows in the Mediterranean region, Mexico, India, Spain and the Middle East [42]. Table 3 shows the classification of anise plant.

Pictures E and F: *Pimpinella anisum* plant and flower, stem and seed respectively.

Table 3: Scientific classification of *Pimpinella anisum*.

Kingdom	**Plantae**
Order	*Apiales*
Family	Umbelliferae
Tribe	*Pimpinelleae*
Genus	*Pimpinella*
Species	*anisum* L.

P. anisum is primarily grown for its seeds (termed aniseeds) that harvested in the period of August and September. The chemical composition analyses showed the major components in the aniseeds are p-anisaldehyde, coumarins, scopoletin,

umbelliferone, sterol, terpene hydrocarbons, polyenes and polyacetylenes. Essential oil mainly contains trans-anethole, phenylpropanoid, and small quantity of estragol, γ-himachalene, *cis*-anethol. The pharmacological studies were performed on aniseed and demonstrated a variety of therapeutic characteristics such as antimicrobial, antifungal, antiviral, antioxidant, inhibition of mucosal damage in stomach, relieving constipation alleviating nausea, hypoglycemic and hypolipidemic effects. Anise seed used as flavoring and appetizer. It has several therapeutic effects from essential oil such as mild expectorant, diuretic, antiseptic, antispasmodic, carminative, relief of gastrointestinal spasms. Consumption of anise in lactating women increases milk and reliefs their infants from gastrointestinal problems and analgesic in migraine [43].

Despite the wide spectrum of pharmacological effects of aniseeds which were proven *in vitro* and *in vivo* tests, very few clinical studies were performed for evaluating the beneficial effects of *Pimpinella anisum* in human models. In 2013, it has been reported that the ethanolic extract of anise seeds exerts significant anticancer effect on prostate cancer cell line (PC-3) compared to normal cells (L6 muscle cell line) and came to the conclusion that it could be helpful in cancer prevention and treatment and may be a natural source of novel anticancer compounds with anti-proliferative and/or apoptotic properties [44]. The extraction followed by HPLC-PDA and Preparative-HPLC were used to fractionate and isolate the most active ingredients in this important plant. Preliminary NMR and MS testing were conducted to determine the structure of the active compound present.

1.5.4. *Ephedra alata* plant

The fourth plant is the *Ephedra alata* Decne. (Alanda in English, العلندى المجنحة in Arabic), is a perennial genus of nonflowering seed herb, the closest living relative of the angiosperm (see picture G) are wild in Palestine. It traditionally used to decrease blood glucose and to control blood pressure, as diuretic, and to relief asthma attack, and the plant stems are frequently chewed for treatment of bacterial and fungal infections. *Ephedra alata* contains mixture of phytochemical as cardiac glycosides, reducing sugars, flavonoid, phenolic compounds and alkaloids [45]. The classification of *Ephedra alata* is shown in the table 4. The *Ephedra alata* is one of the species in *Ephedra* L a genus of gymnosperm shrubs from Ephedraceae family. The ephedra shrub is nearly leafless, jointed with green to brown stems that stands 30.48 cm high,

on average but it may grow up to 121.92 cm The various species of Ephedra are widespread in many lands, native to north America, southern Europe, northern Africa, Arab world region, and the Mediterranean area. Ephedra also known as ma huang, used in traditional Chinese medicine to treat asthma or other lung problems [46]. Its species contain alkaloids of biological relevance like ephedrine, pseudophedrine, norephedrine, norpseudoephedrine (cathine), methylephedrine and methylpseudoephedrine. While these effects are usually harmless to healthy individuals, caution is needed for those who suffer from heart disease or hypertension or diabetes or hyperthyroidism [47].

Picture G: The *Ephedra alata* plants grows wild in West Bank-Palestine.

Table 4: Scientific classification of *Ephedra alata*.

Kingdom	**Plantae**
Division	Gnetophyta
Class	Gnetopsida
Order	Ephedrales
Family	Ephedraceae
Genus	*Ephedra*
Species	*E. alata*

Alanda water extract herb has recently been used to treat bladder cancer which spread all over the body of a farmer from Jenin. He returns its importance in preventing the spread of cancer cells and their ability to combat cancer of all types. Therefore, the

plant was subjected to extraction by methanol, followed by preparative and analytical HPLC-PDA to acquire fractionation for biological assay. To study the toxicity for *in vitro* test, screening the crude methanolic extract of *E. alata* against HepG2 (is a perpetual cell line consisting of human liver carcinoma cells, derived from the liver tissue). The HepG2 cell line was originally established in 1979 by Barbara Knowles and colleagues, and mistakenly reported as a hepatocellular carcinoma [48].

1.6. Analytical methods for the analysis of plant constituents

1.6.1. High Performance Liquid Chromatography (HPLC)

Liquid Chromatography (LC) is the most widely used of all the analytical separation techniques. The reasons for popularity of the method are include sensitivity, adaptability to accurate quantitative determinations, ease of automation, and suitability for separating nonvolatile species or thermally fragile one based on differential rates of migration of analytes through a stationary phase by movement of liquid mobile phase. Pumping pressures of several hundred atmospheres are required to achieve reasonable flow rates with packing of 3 to 10 µm, which are common in modern LC. Because of these high pressures, the equipment for HPLC tend to be more elaborate and expensive than equipment for other type of chromatography. It's a widespread applicability of organic and inorganic substance [49].

1.6.2. Gas Chromatography - Mass Spectroscopy (GC-MS)

GC-MS is a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS). GC can separate sample compounds with high resolution, While MS can provide detailed structural information on most compounds such that they can be exactly identified and quantified [50].

Gas Chromatography where the volatile or Semi-volatile sample vaporized by injection into a heated injector, eluted through a capillary column (stationary phase) by inert gaseous mobile phase (carrier gas such as helium or nitrogen) and gradient. Temperature and finally detected by detector separation is based on differences in boiling points of the solutes and the solutes interaction with the stationary phase (compounds with low boiling points elute faster than those with higher boiling points). Separated compounds elute from the column and enter a detector, which is

capable of creating an electronic signal in response to the presence of a compound, the signal size is proportional to the concentration of the compound in the sample. At the end, signal is processed by a computer.

Mass Spectroscopy is an analytical technique that utilizes the degree of deflection of charged particles by a magnetic field to find the relative masses of molecular ions and fragments. It's a powerful method because it provides a great deal of information and can be conducted on tiny samples. Mass spectrometry has a number of application in organic chemistry, including determining molecular mass, providing data on isotopic abundance [51,52]. A mass spectrometer generates multiple ion from the sample under investigation, it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type.

Fig. 5 shows schematic of hyphenated GC-MS. It usually consist of single quadrupole mass analyzer or ion trap. Normally, it used electron multiplier as detector, and all MS systems need ion source, either electron impact (EI) hard ionization, or soft chemical ionization (CI-positive or negative), [53].

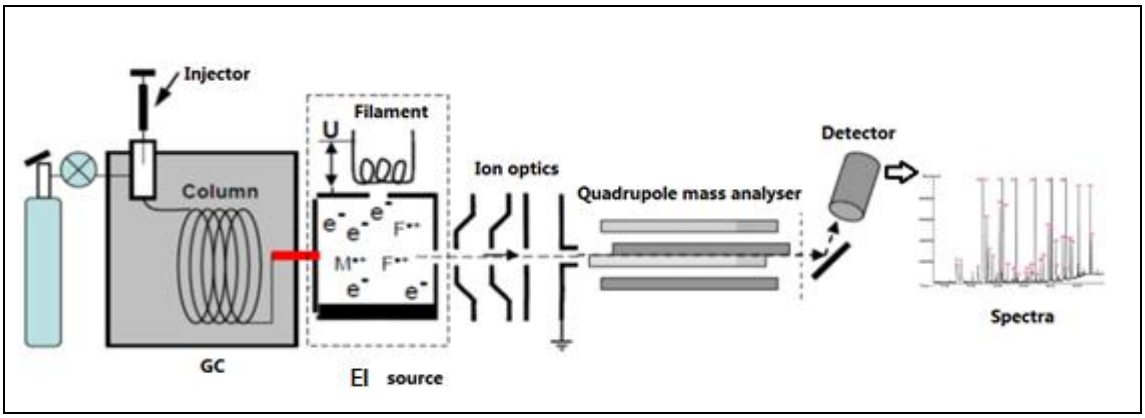


Fig. 5: Schematic diagram of quadrupole GC-MS.

1.7. Research problem

In view of the fact that chemotherapeutic drugs are very effective but unselective, severe side effects like weakened immune system limit the use of these drugs. People have to pay more attention to natural sources as alternatives to treat cancer such as the use of plant-derived products that may have reduced adverse side effects [54].

Moreover, plants and other natural sources were for a long time the main source of anti-tumor drug candidates [55].

1.8. Aim of the study

The aim of this study, is to screen four Palestinian plants to assess the antiproliferative activity or growth inhibition of various human cancer cell line, to determine the bioactive constituent through techniques to know the structures of compounds.

1.9. Objective of the study

1. Screening of the natural plants, which include *Cuscuta palaestina, Gundelia tournefortii, Pimpinella anisum, and Ephedra alata* crude extracts using different solvents and to explore their cytotoxic effect on human colon cancer cell line (HCT-166), human prostate cancer cell line (PC-3), and human liver cancer cell line (HepG2).
2. To test the *in vitro* cytotoxicity for the cancer cells based on the determination of EC_{50} values of the extracts by using the MTT and LDH leakage assay.
3. To utilize the preparative HPLC coupled with photodiode array detector to separate different fractions in the reversed phase mode, and to test the *in vitro* cytotoxic assay of each fraction, then to try to isolate the active pure compounds from active fraction along with the assay testing.
4. To analyze and fully characterize the active compounds by using GC-MS, High Resolution-Mass Spectrometry (HR-MS), and ^{1}H-NMR.

1.10. Question of the study

1. What are the main bioactive compounds present in the natural plant, and their identities?
2. What are their biological assay activities *in vitro* test?

1.11. Hypotheses

The bioactive compound isolated from natural Palestinian plants affected the growth of cancer disease.

Chapter Two

Literature Review

2. Literature Review

Cuscuta palaestina Boiss were collected and studied from eastern Crete near Sitia. It contained some alkaloids such as sparteine, 11,12-dehydrosparteine, retamine, *N*-methylcytisine, cytisine, 17-oxosparteine. These alkaloid were isolated by GC-MS [56]. Plants have to defend themselves against herbivores and microorganisms by production of chemical defense compounds or allelochemicals [57]. Up to current, there are neither data in the literature concerning the analysis of anti-proliferative and anticancer activity in *Cuscuta palaestina* nor chemical composition of their active constituents.

Gundelia tournefortii is a well-known medicinal plant in Eastern Anatolia. Antioxidant activities of *G. tournefortii*, were investigated and the results revealed that the seeds have higher antioxidant potential than the aerial parts [58]. The *Gundelia tournefortii* have a physiochemical properties of seed oil content and the saponification value of its oil were 22.8% and 166.05 respectively. Oleic and linoleic acid content of oil were found 27.99% and 54.59% respectively [59]. Intensive search via PUBMED is shown in Fig. 6. There is a noticeable increase in the number of publications after the year 2000. Up to current, there is no single report on the effects of *Gundelia tournefortii* extracts against cancer.

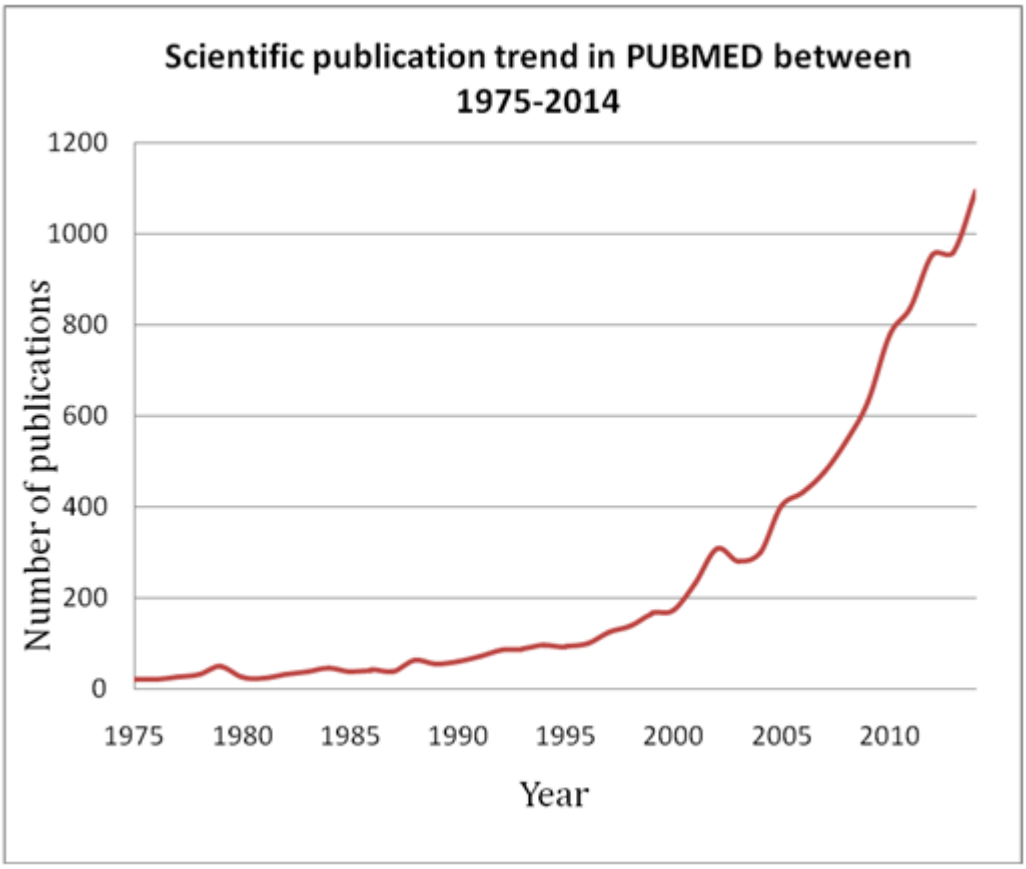


Fig. 6: Publication trend analysis revealed a sharp increase in scientific publications especially after 2000 related to the area of natural product drug discovery. The keywords for our search in PUBMED were "Natural/ Products/ Drug/ Discovery". The search was done in 13 November 2015. As shown in the figure, the year 2000 could be the turning point.

However, the effect of *Gundelia tournefortii* on some biochemical parameters in hyperglycemic and hyperlipidemic mice male albino mice were conducted and found to induce hyperglycemic and hyperlipidemic by daily injection of dexamethasone were the *G. tournefortii* extract as hypoglycemia and hypolipidemia in dexamethasone treated mice [60].

The anticancer activity of ethanol extract of anise *(Pimpinella anisum)* seed was investigated. MTT and LDH assays revealed that ethanolic extract have cytotoxic activity on human prostate cancer cell line [PC-3] at concentration found safe to normal cells (rat skeletal muscle cell line [L6]). Treatment with anise seed extract caused anti proliferative and apoptotic effects, with IC_{50}value of 400µg/ml to cancer cell. Thus, anise could be one of the foods that attribute to cancer prevention and

treatment. It is worth to assign that anise seeds could contain more than one chemical active against cancer cells via targeting, as well to isolate and identification of novel anticancer drug candidates from aniseed [44].

The essential oil of fruit *Pimpinella anisum* obtained by hydrodistillation, were analyzed by GC-MS. The oil of aniseed was characterized by higher amount of trans-anethole (96.80%), and the isomer of trans-anethole methyl chavicol (estragol) 0.19%. Apart from these two phenylpropanoids, nine sesquiterpenes and one monoterpene were present in essential oil of Pimpinella anisum which constituted the other 3.01% of essential oil but only γ-himachalene was present with more than 1% [61]. The aniseed have anti-microbial activity against Gram positive (Bacillus subtilis) and Gram negative (*E. Coli*) [62].

Ephedra alata methanolic extract showed that it has high antioxidant activity, and the IC_{50} for the plant was almost equivalent to the Trolox standard antioxidant which justified its uses in the Palestinian traditional medicines [45]. The *in vitro* study was conducted and aims to evaluate cytotoxic and cytostatic effects of *E. alata* water-ethanolic extract by using MTT assay and LDH assay. The cytostatic activity was more potent in co-culture with IC_{50} of 380 µg/ml against HepG2 cell line, so the *E. alata* traditionally known for anticancer effect [60]. The aerial parts of different *Ephedra* species containing 0.02% to 3.4% of 6 optically active alkaloids, which include (-)-Ephedrine, (+)-Pseudoephedrine, (-)-N-Methylephedrine, (+)-N-Methylpseudoephedrine, (-)-Norephedrine, (+) Norpseudoephedrine were investigated [64]. Reliable method was used to determine ephedrine alkaloids and 2,3,5,6,-tetramethylpyrazine (TMP) in *Ephedra sinica* by GC- MS [65]. Some Ephedrine type alkaloid content was used as nutritional and determined by HPLC from *Ephedra sinica* (Ma-huang) [66].

Chapter Three

Methodology

3. Methodology

3.1. Materials, and collection Plant

- Approximately 30 g of *Cuscuta palaestina* plant**,** which comprises stems and flowers, was collected from farms near the city of Taiba, in the Triangle. The specimens were washed with distilled water and dried in the shade (see picture H)**.** Supernatant obtained was passed through a 0.2 µm filter for both methanol and hexane extract.

Picture H: Dried *Cuscuta palaestina* plant used for extraction in the current study.

- 30 g of the aerial parts (stem, leaves and the head) of *Gundelia tournefortii* were collected from the mountains of Nablus (northern Palestine), during February-March, 2014. The specimens were dried in the shade at room temperature. The Pictures below depicts the plant before and after removing its prickles.


Pictures I and J: *Gundelia tournefortii* plant growing wild in Palestine before and after removing the prickles.

- Anise seeds were from (Al Alim - Medicinal Herb Center, Zippori), approximately 15 g.
- 10 g of *Ephedra alata* aerial parts were collected in spring and summer 2014 from the mountains of Jenin region of West Bank / Palestine. The plants were dried for 7-10 days in the shade at environmental temperatures. The dried plants were then ground and stored in cloth bags at 5 ˚C until their transfer to the laboratory for preparation of the plant extracts.

Picture K: the stem of ephedra collected after dried in shade of the lab.

- The HCT-116 cell line (human colon cancer) were purchased from American-type culture collection (ATCC) (Manassas, VA) to be use in the *in vitro* test.
- The MTT dye were purchased from Millipore Cat.# CT02. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide).

- Purchased 50 unit of Cells (2×10^4/well) were plated in 200 µl, and 100 µl of medium/well in 96-well plates (corning incorporated, USA).
- PC-3 cell line (human prostate cancer) were purchased from American Type Culture Collection (ATCC) (Manassas, VA) though their representative in Israel, Biological Industries Israel Beit Haemek Ltd. The cells were grown in F-12K (ATCC) (PC-3 cells) medium, were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C.
- The HepG2 cell line (human liver cancer) were from ATCC repository (American Type Culture Collection, Rockville, MD, USA), to be use *in vitro* test.
- 10 mg of sesamin analytical standard (59867-10 MG, P Code 10156745, sigma-Aldrich).

3.2. Reagents

- Solvent such as Methanol, Hexane, DMSO, Propanol, Ethanol, Ethyl acetate, Chloroform, Isopropanol, Acetic acid, HCl were from Sigma-Aldrich (Jerusalem).
- Acetonitrile and water are all HPLC grade were from Sigma Aldrich (Jerusalem).
- The *N,O*-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane used for GC silylation derivatization (>99%, Sigma-Aldrich)

3.3. Instrumentation system

- Hyphenated GC-MS consists of Agilent Technologies 7890A GC system coupled with a mass spectrometer of Agilent Technologies 5975C inert MSD with Triple-Axis Detector. GC-MS was operated in the electron impact ionization mode (EI) at 70 eV.
- HP7890 gas chromatograph coupled to HP5973 mass spectrometer (electron multiplier potential 2KV, filament current 0.35 mA, electron energy 70 eV, using the splitless injection mode. An Agilent 7683 auto-sampler was used for the samples introduction. A 15 m, 0.32 mm ID 5% cross-linked phenylmethyl siloxane capillary column (HP-5MS) with a 0.25 µm film thickness was used for separation.
- Analytical High Pressure Liquid Chromatography (HPLC-PDA) system consists of an alliance 2695 HPLC, 2998-Photo diode array (PDA), Empower^3 software.

- Preparative High Pressure Liquid Chromatography (Prep-HPLC-PDA), Empower PDA Software, system consists of Water^{TM} 600 Controller HPLC (3535 quaternary gradient module), and Water^{TM} 996 Photodiode Array Detector, and the Cosmosil Packed column (Code No: 3802401, 5C18-MS-II, Size 20 ID x 250 mm, Manf. No. K60973) and pre-column (Cosmosil Guard Column, 5C18-MS-II, Size 20 ID 20 mm, Manf. No. K62526).

3.4. Equipment's

- Analytical Balance (Sartorius, accuracy ±0.0001g, Germany).
- Ultrasonic bath SW12H internal 300 mm x 240 mm x 200 mm (w x d x h).
- Rotary evaporator RE-3000A was used, 220 V, 50 Hz capacity 1000 ml, receiving bottle 1000 ml, speed 20-200 r/min, temp 25 - 90 C$^{o.}$
- Whatman 6900-2502 GD/X 25 Sterile Syringe Filter, 25 mm, 0.2 Micron, PVDF Filtration Medium, Pack of 50.
- The GC was operated on an Agilent J&W GC column HP-5 column (30 m x 0.32 mm, id. with 0.25 µm film thickness).
- Laboratory glassware (volumetric flasks, measuring cylinders, beakers, volumetric pipettes, and graduated pipettes).
- Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and PTFE / silicone Septum,100 / pkg.
- Remedil Disposable syringe 2.5 ml, LOT NO 120308.
- Acrodisc® syringe filters Nylon membrane, diam. 13 mm, pore size 0.2 µm.

3.5. Extraction Methods and analytical procedures

3.5.1. *Cuscuta palaestina* plant

- **Procedure of the sample extract and chromatographic conditions**

Cuscuta palaestina: 250 ml of each methanol and hexane were added to the dried ground plant material (25 g) after separated in a tow beakers, and the samples were sonicated for 120 minutes at 60 °C, then left in a dark glass bottle for 24 h for complete extraction. The methanol extract concentrated by a rotary vacuum evaporator under reduced pressure and the hexane extract was evaporated to dryness under pressure at 50 °C and dissolved in DMSO for *in vitro* studies. The dry yield of

the extracts was 3.6 gr (14.4%) and 0.46 gr (1.8%) for methanol and hexane extracts, respectively. Supernatant obtained was passed through a 0.2 µm filter.
A 10 mg of Sesamin standard was dissolved in 40 ml of ethanol.

- **Calibration Curve of standards**

Sesamin stock solution was prepared by dissolving 10 mg of sesamin standard in 40 ml ethanol, this brings about 250 ppm solution., The UV-Vis of sesamin was stored in the library of HPLC-PDA for matching and quantitation of sesamin in samples., Several dilution was made by using the dilution equation $M_1\ V_1 = M_2\ V_2$ to prepare 25 ppm, 62.5 ppm, 125 ppm and 187.5 ppm., The solutions were filtered using 0.45 µm membrane filters. The calibration curve was constructed and the linear range was determined. The linearity was evaluated by linear regression analysis and the R^2 value was projected. Limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (*S/N*) of 3 and 10, respectively.

- **Preparation of extracts from different solvents**

Solution of *Cuscuta Palaestina were* prepared included 0.9 mg/ml in hexane, 9.7 mg/ml in methanol, 2.5 mg/ml in ethanol, and 1mg/ml in chloroform.

- **Sesamin in *Cuscuta palaestina* or from host plant.**

Different extracts in methanol with two concentration were prepared as shown in Table 5. Five extracts of *Cuscuta Palaestina* that are parasite on some plants like *Malva sylvestris* (الخبيزة in Arabic), *Cichorium Intybus* (علّتْ in Arabic), *Prosopis farcta* (يَنْبوت in Arabic), *Portulaca oleracea* (بَربَزينة in Arabic), and unknown *X*. Another five extracts from host plants alone were injected using the same chromatographic conditions into analytical HPLC-.

Table 5: The concentration of extract in methanol for different plants with *Cuscuta palaestina* as parasite on host plant, and host plant alone.

No	Plant	Conc. (mg/ml)	Conc. (mg/8 ml)
1	*Cuscuta palaestina* + *Malva sylvestris*	3.9375	31.5
2	*Cuscuta palaestina* + *Cichorium Intybus*	3.275	26.2
3	*Cuscuta palaestina* + *Prosopis farcta*	3.525	28.2
4	*Cuscuta palaestina* + *Portulaca oleracea*	2.7375	21.9
5	*Cuscuta palaestina* + *X* (unknown plant)	3.8875	31.1
6	*Malva sylvestris*	6.4875	51.9
7	*Cichorium Intybus*	7.7	61.6
8	*Prosopis farcta*	2.5625	20.5
9	*Portulaca oleracea*	3.8625	30.9
10	*X* (unknown plant)	7.925	63.4

3.5.2. *Gundelia tournefortii* plant

- **Procedure of the sample extract**

The air-dried aerial parts of *Gundelia tournefortii* were finely powdered. Three equal fractions weighting 25 ± 0.01 g of the ground plant was then transferred into Erlenmeyer flasks were then extracted with 250 mL of Methanol (H_3COH), Water (H_2O) or Hexane (C_6H_{14}) solvents. The flasks was then sonicated for 2 h at 50 ^oC, left in dark glass bottles for 24 h for complete extraction. The hexane extract was filtered and evaporated to dryness under pressure at 50°C and dissolved in DMSO for *in vitro* experiments. The methanolic extract was filtered and dried by a rotary evaporator under vacuum. The yield of the extracts was found to be 3.2%, 1.8% and 12% (wt/wt) for Methanol, Water and Hexane extracts, respectively. The stock crude extracts were preserved in an airtight glass container and kept at -20 °C until use.

- **Fractionation by Preparative HPLC-PDA**

Eight fractions were collected using prep-HPLC-PDA in order to test it *in vitro*. 47 mg extract was dissolved in 20 ml methanol, 1ml of ethanol and 1 ml of acetonitrile, shaked well and filtrated fooled by injection to the Preparative HPLC.

3.5.3. *Pimpinella anisum* plant

- **Procedure of the sample extract**

3 gram of seeds of Pimpinella anisum (anise) was washed with distilled water and dried in shade. It was finally grinded to powder then added to 15 ml of methanol in a beaker with magnetic stirrer and homogenized for 7 minutes at 50°C. Then left in dark glass bottle for 24 h for complete extraction. The extract supernatant obtained was passed through a 0.2 m filter, and methanol was evaporated to give 2.5 gram of crude extract.

- **Fractionation by prep-HPLC-PDA**

One gram of crude *Pimpinella anisum* was dissolved in 10 ml of 1% acetic acid solution (100 mg/ml), and was filtered through 0.45 µm membrane filter before injection. One ml of dark clear solution was directly injected to the prep-HPLC and six fractions were collected. Evaporate the solvent of each fraction through the rotary evaporator.

3.5.4. *Ephedra alata* plant

- **Procedure of the sample extract**

2 g of the hand grinded of *ephedra* plant, the material were added to 30 ml of methanol and boiled for 7 minutes under stirring. The extract supernatants obtained were passed through a 0.2 mm filter, and freeze dried. Take 1000 µg/ml of the crude extract for further evaluation for *in vitro* test.

- **Fractionation by prep-HPLC-PDA**

25 mg/ml methanolic extract of *ephedra* plant was directly injected into the prep-HPLC-PDA.

3.5.5. GC-MS analysis conditions for *Cuscuta palaestina*

Components of *Cuscuta palaestina* from methanol and hexane extracts were ran and identified by using GC-MS of Agilent Technologies 7890A GC system coupled with a mass spectrometer of Agilent Technologies 5975C inert MSD with Triple-Axis Detector. The GC was operated on an Agilent J&W GC column HP-5 column (30 m x 0.32 mm, id. with 0.25 μm film thickness). The carrier gas was helium at a flow rate of 1.2 ml/minute and the injection volume was 1μl. Injection port temperature was 300 °C and the ionization voltage was 70 eV. The samples were injected in split mode of 10:1. Mass spectral were recorded every 1 second scan over a range set at 45-800 (m/z).The oven temperature program was started with an initial temperature of 50 °C and held for 5 minutes, then ramped to 320 °C with a heating rate of 5 °C/min and finally held for extra 20 minutes. The injection port temperature was 280°C and the MS interface temperature was 300°C.Solvent delay time was 7 minutes to get rid of the gigantic solvent peak. The obtained mass spectra were preliminarily interpreted by comparing with the Mass Spectral Library of the National Institute of Standards and Technology (NIST, Gaithersburg, USA).

3.5.6. GC-MS analysis conditions for *Gundelia tournefortii*

Derivatized samples of *Gundelia tournefortii* extracts GC-MS analysis were carried out using HP7890 gas chromatograph coupled to HP5973 mass spectrometer (electron multiplier potential 2KV, filament current 0.35 mA, electron energy 70 eV, and the spectra were recorded every second over the range of (m/z) 50 to 800 Da using the splitless injection mode. An Agilent 7683 auto-sampler was used for the samples introduction. A 15 m, 0.32 mm ID 5% cross-linked phenylmethyl siloxane capillary column (HP-5MS) with a 0.25μm film thickness was used for separation. Helium was the carrier gas at a constant flow of 1.1 mL/minute. An isothermal temperature was first held at 50 °C for 2 minutes, followed by a ramp gradient of 10 °C per minute to 320 °C and held there for 10 minutes. The total GC-MS run time was 39 minutes. The injection port temperature was 220 °C and the MS interface temperature was 300 °C. Solvent delay time was 8 minutes to eliminate the gigantic hexane and methanol solvent peaks.

Underivatized samples of *Gundelia tournefortii* Solutions from methanol and hexane extracts were ran and identified by using GC-MS of Agilent Technologies 7890A GC

system coupled with a mass spectrometer of Agilent Technologies 5975C inert MSD with Triple-Axis Detector. The GC was operated on an Agilent J&W GC column HP-5 column (30 m x 0.32 mm, id. with 0.25 μm film thickness). The carrier gas was helium at a flow rate of 1.2 mL/minute and the injection volume was 1 μL. Injection port temperature was 300 °C and the ionization voltage was 70 eV. The samples were injected in the split mode with a ratio of 10:1. Mass spectra were recorded every second over a range set at (m/z) of 45-800 Da. The oven temperature program was started with an initial temperature of 50 °C and held for 5 minutes, then ramped to 320 °C with a heating rate of 5 °C/min and finally held for extra 20 minutes. The total GC-MS run time was 79 minutes and solvent delay time was 7 minutes. The injection port temperature was 280 °C and the MS interface temperature was 300 °C. Identification of the phytochemical components, the percentages of the phytochemical components were computed from the GC peak areas by normalization. Library searches were carried out using the Mass Spectral Library of the National Institute of Standards and Technology (NIST, Gaithersburg, USA) or with mass spectra extracted from the literature.

3.5.7. Chromatographic conditions of HPLC-PDA

3.5.7.1. Analytical HPLC-PDA

- **Method to quantitative of *Cuscuta palaestina* of standards and samples.**

Chromatographic conditions used to separate and quantify sesamin in *Cuscuta palaestina*. Crude sample was run on reversed phase ODS column of Waters (XBridge, 4.6 ID x 150 mm, 5 μm) with guard column of XBridge ODS (20 mm x 4.6mm ID, 5 μm). The mobile phase consisted of binary solvent mixture of 0.5% acetic acid solution (A) and acetonitrile (B) in linear gradient mode. A 100% A was initially used and then descended to 70% A in 40 minutes, then to 40% A in 20 minutes, finally to 10% A in 2 minutes and stayed there for 6 minutes and then back to the initial conditions in 2 minutes. The HPLC system was equilibrated for 5 minutes with the initial acidic water mobile phase (100 % A) before injecting next sample. All the samples were filtered with a 0.45 μm PTFE filter. The PDA wavelengths range was from 210-500 nm. The flow rate was 1 ml/min. Injection volume was 10 μl and the column temperature was at room temperature.

3.5.7.2. Prep-HPLC-PDA

- **Method to separation of *Gundelia tournefortii* sample**

Chromatographic condition used to collect different fraction was a gradient combination of eluent A (Milli-Q water); eluent B (acetonitrile). injection volume of 1 ml. Flow rate was 20.0 ml/min and the gradient elution of the gradient method is 95.0% A and 5.0% B for 0-2 minutes, then a linear gradient of 95.0% A and 5.0% B for 2-18 minutes, then a linear gradient of 0.0% A and 100.0% B for 18-19 minutes, then a linear gradient to 0.0 %A and 100.0 % B for 19-20 minutes, then a linear gradient to 95.0% A and 5.0% B for 20-22 minutes.

- **Method to separation of *Pimpinella anisum* sample**

The prep-HPLC experiments were run on ODS column (Agilent Prep-HT C18, 22.2 x 250 mm, 10 μm). The eluent A was highly pure water (Milli-Q) and acetonitrile (B) was programmed for a linear gradient starting with 50% A and directly raised to 10% A in 18 minutes and stayed there for 1 minute and get back to 50% A in 1 minute,. The HPLC system was equilibrated 4 minutes with the initial mobile phase before injecting next sample. All the samples were filtered with a 0.45 μm micro porous filter. Flow rate which was 20 ml/minutes, the injection volume was 1 ml and the column temperature was set at room temperature.

- **Method to separation of *Ephedra sample***

The prep-HPLC-PDA used to run sample of ephedra was by a gradient elution A (Milli-Q water); eluent B (acetonitrile). Injection volume was 1 ml and the mobile phase flow rate was flow 15.0 ml/min. The gradient elution was 98.0% A and 2.0% B for 0-2 minutes, , then 98.0% A and 2.0% B from 2-20 minutes, then 20.0% A and 80.0% B form 20-22 minutes, 20.0% A and 80.0% B from 22-23 minutes and then returned back to 98.0% A and 2.0% B in one minute. 10μl of *Ephedra alata* methanolic extract was injected into analytical HPLC-PDA.

3.5.8. Cell cultures and treatments

Cell line HCT-116 (human colon cancer) were maintained as adherent cells in DMEM supplemented with 10 percent heat-inactivated fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin at 37° C in humidified 5% CO_2 and a 95% air

atmosphere. The medium was refreshed every 2 or 3 days, and HCT-116 cells were trypsinized by 0.5 percent trypsin–EDTA when the cells reached 80% to 90% confluence. The well-grown cells were harvested and seeded in 96-well plates at a density of 2×10^4 cells/200 µL and cultured overnight before treatment.

PC-3 cells (human prostate cancer cell line). The cells were grown in F-12K (ATCC) (PC-3 cells) medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 0.1 mg/ml streptomycin. All cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37ºC.

The HepG2 cell line (human liver cancer) was grown in DMEM-5671 medium, with a high glucose content (4.5 g/l), supplemented with 10% vol inactivated fetal calf serum (FCS), 1% nonessential amino acids, 1% glutamine, 100 U/ml penicillin, and 10 µg/ml streptomycin (sigma Aldrich) at 37° C in humidified 5% CO_2 and a 95% air atmosphere. For the cytotoxic assay, 20,000 cells/100 µL medium were seeded in 96-microplates.

It is important to mention that all the anticancer part in this thesis was done in a collaborative work with Professor Anwar Rayan, Qasemi Research Center, Al-Qasemi Academic College, Baka EL-Garbiah.

3.5.9. Micro-culture tetrazolium assay (MTT)

In general MTT colorimetric assays are used extensively in cell proliferation and cytotoxicity, which are based upon the bioreduction (reduced by metabolically active cell) of a yellow water soluble tetrazolium salt to purple water insoluble formazan by mitochondrial dehydrogenase of living cells in which tetrazolium ring gets cleaved in mitochondria as shown in Fig. 7. The MTT assay, developed by Mosmann and described for the first time by him, depends on the reduction of tetrazolium salt by living cells.

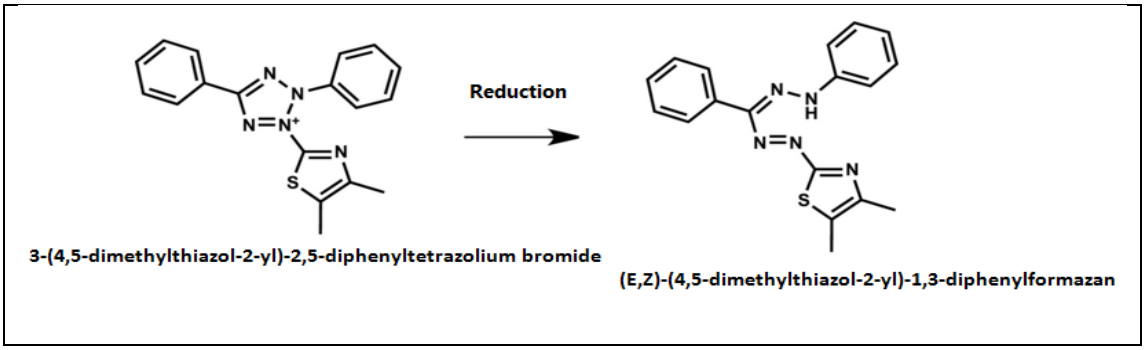


Fig. 7: MTT reduced by metabolically active cell to Formazan.

Cuscuta palaestina methanol and hexane extracts were added to the cells at increasing concentrations (0-1000 µg/ml) by performing the micro-culture tetrazolium (MTT) assay [67], based on the capacity of mitochondrial succinate dehydrogenase enzymes in living cells to reduce the yellow water-soluble substrate (MTT) into an insoluble colored formazan product, which can be measured spectrophotometrically. The monolayer cell culture was trypsinized, and the cell count was adjusted using a medium containing a 10% fetal calf serum (FCS). After 24 h, when the monolayer formed, the supernatant was flicked off, and 100 µl of methanol and aqueous extracts were added to the cells in microtiter plates separately and kept for incubation at 37° C in a 5 percent CO_2 incubator for 24 h, during which time the cells were periodically checked for granularity, shrinkage, and swelling. After 24 h, the sample dilution in the wells was flicked off, and 100 µL of the MTT dye was added to each well. The plates were gently shaken and incubated for 4 h at 37° C in the 5 percent CO_2 incubator. The supernatant was removed, 100 µL of propanol was added, and the plates were gently shaken to solubilize the formed formazan. Absorbance was measured using a microplate reader at a wavelength of 620 nm [68,69]. All experiments were repeated in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

$$\text{Percent viability} = \left(\frac{A_{620\ \text{nm}}\ \text{of plant extract treated sample}}{A_{620\ \text{nm}}\ \text{of nontreated sample}}\right) * 100$$

For *Gundelia tournefortii*, and *ephedra alata* the same method of MTT assay was adopted, a cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product accumulates only in healthy cells. The assay was optimized for the cell lines used in the experiments. MTT was applied to assess cell viability as described in a previous report [70]. Cells (2×10^4/well) were plated in 200 µl of medium/well in 96-well plates and were allowed to attach to the plate for 24 h. Plant extracts were added at increasing concentrations (0-1 mg/mL) for 22 h. The cell medium was then replaced with 100 µl fresh medium/well containing 0.5 mg/mL MTT and cultivated for another 4 h darkened in the cells incubator. The supernatant was removed and 100 µl isopropanol/HCl (2% HCl (0.1 M) in isopropanol) were added per well. The absorbance at 620 nm was measured with micro-titer plate reader

(Anthos). Two wells per plate without cells served as blank. All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

$$\text{Percent viability} = \left(\frac{A_{620\ \text{nm}}\ \text{of plant extract treated sample}}{A_{620\ \text{nm}}\ \text{of nontreated sample}}\right) * 100$$

The *Pimpinella anisum* MTT assay is a cell viability test often used to determine cytotoxicity following exposure to toxic substances. Cells (2x10^4/well) were plated in 100 µl of medium/well in 96-well plates and were allowed to attach to the plate for 24 h. *Pimpinella anisum* seeds' extract was added at increasing concentrations (0-1 mg/ml) for 24 h. The cells medium was replaced with 50 µl fresh medium/well containing 0.5 mg/ml MTT and cultivated for another 4 h darkened in the cells incubator. The supernatant was removed and 50 µl isopropanol/HCl (1mM HCl in 100% isopropanol) were added per well. The absorbance at 570 nm was measured with microplate reader (Anthos). Two wells per plate without cells served as blank. All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula

$$\text{Percent of viability} = \left(\frac{A_{570\ nm}\ \text{of plant extract treated sample}}{A_{570\ nm}\ \text{of plant extract non treated sample}}\right) * 100\%$$

3.5.10. Silylation derivatization

One ml of *Gundelia tournefortii* extracts was taken for each extract was transferred to a 2-mL glass vial, and the accumulated solvents were evaporated under a gentle stream of nitrogen at ambient temperature. A 150.0 µL of *N,O*-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane reagent used for GC silylation derivatization (>99%, Sigma-Aldrich) was added to each dry *Gundelia tournefortii* crude extract followed by heating up to 70 °C for 20 minutes [71]. One µL of each derivatized sample was injected into the gas chromatograph (GC) coupled with mass selective detector (MS).

3.5.11. Lactate dehydrogenase (LDH) leakage assay

This is a quantitative colorimetric assay that measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay, which results in

the conversion of a tetrazolium salt (INT) into a red formazan product. All experiments were conducted in triplicates. For *Cuscuta palaestina* extracts take 50µl from well plat before added MTT assay, and transfer to another 96-well plate to determine the percentage of LDH released in relation to the control was calculated as follows:

$$\text{Percent viability} = \left(\frac{A_{492\ \text{nm}}\ \text{of plant extract treated sample}}{A_{492\ \text{nm}}\ \text{of nontreated sample}}\right) * 100$$

For the *Pimpinella anisum* the permeability of cellular membranes following the exposures was determined by measuring the amount of released lactate dehydrogenase (LDH) enzyme from PC-3. Activity of LDH released to the cell culture medium was monitored following the formation of formazan by coupled enzymatic reaction at 500 nm according to the manufacture kit (CytoTox 96, Promega). Cell membrane rupture was defined as the ratio of LDH activity in the supernatant of treated cell to the LDH activity released in the control cells. PC-3 cells (2x10^4/well) were plated in 50 µl of medium/well in 96-well plates and were allowed to attach to the plate for 24 h. After cell attachment (24 h) cells were treated with increasing concentrations of the plant extracts (0-1 mg/ml). The extracellular LDH activity was measured in the medium after 24 h. Therefore, 50 µl from each well was transferred to a new 96 well plate; the enzyme reaction was carried out according to the manufacture kit. All experiments were repeated three times in triplicates. The effect of the plants extracts on cell viability was expressed using the following formula:

$$\text{Percent of viability} = \left(\frac{A_{500\ nm}\ \text{of plant extract treated sample}}{A_{500\ nm}\ \text{of plant extract non treated sample}}\right) * 100\%$$

Chapter Four

Results and Discussion

4. Results and Discussion

4.1.*Cuscuta palaestina* plant

4.1.1. Analysis by GC-MS

The phytochemical screening using GC-MS methodology revealed 18-components identified in the methanol extract of *Cuscuta palaestina* for the first time. Reasonably, less compounds were noticed in the hexane extract. Good resolution was obtained in both chromatograms since a 79-minute analysis scan run was adopted ending up with a high temperature of 320°C (see the experimental section). This would facilitate the elution of high molecular weight compounds out of the capillary GC HP-5 column. Figures 8 and 10 show the total ion chromatogram of methanol and hexane extract injections respectively. Dodecanoic acid isooctyl ester (20.96%), Palmitic acid (10.58%), 2-Fluoro-3-trifluoromethylbenzoic acid, heptadecyl ester (6.7%) and 2-Fluoro-5-trifluoromethylbenzoic acid, heptadecyl ester (6.88%) are the major compounds in the methanol extract. The major chemicals in the hexane extract are 8-Hexylpentadecane (54.66%), 9-Octylheptadecane (9.33%) and trans-3,4-Dimethyl-2-pentene (8.42%). The main components along with their retention time (RT) and peak area percentages are presented in Tables 6 and 7. Figures 9 and 11 shows the chemical structures of the major components in the methanolic and hexane extracts, respectively.

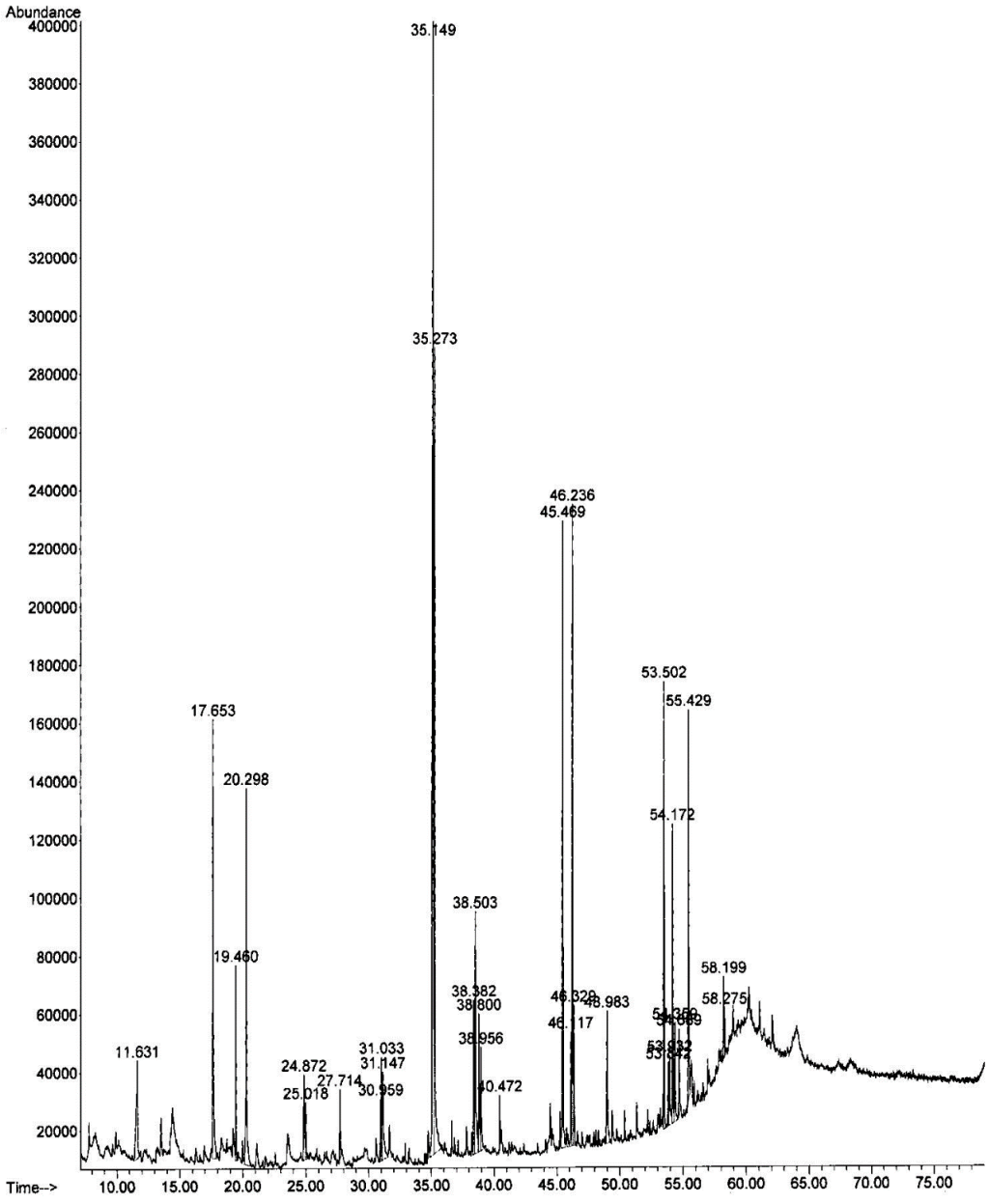


Fig. 8: GC-MS analysis of methanol extract of *Cuscuta palaestina*.

Table 6: Components of *Cuscuta palaestina* methanolic extract verified by GC-MS. Three phytochemicals namely Sesamin, Campesterol and Stigmasterol are bolded and could be the source for the methanolic extract anticancer activity.

No.	Compound name	RT (minutes)	% Peak Area
1	Propanoic acid	11.631	2.619
2	2-Methylbenzaldehyde	17.653	5.376
3	1-Formylpyrrolidine	19.460	1.754
4	2-Methoxy-4-vinylphenol	20.298	4.930
5	1,6-Anhydro-β-D-glucose	24.872	1.543
6	Hexanoic acid	27.714	1.087
7	Dodecanoic acid isooctyl ester	35.149	20.965
8	Palmitic acid	35.273	10.582
9	(Z,Z)-9,12-Octadecadienoic acid	38.382	1.755
10	Palmitoleic acid	38.503	3.915
11	Oleic acid	38.800	1.860
12	2-Fluoro-3-trifluoromethylbenzoic acid, heptadecyl ester	45.469	6.700
13	2-Fluoro-5-trifluoromethylbenzoic acid, heptadecyl ester	46.236	6.875
14	**Sesamin**	**53.502**	**4.490**
15	ethanone, 2-(3H-imidazo[4,5-b]pyridin-2-ylthio)-1-(4-morpholinyl)-	54.172	3.222
16	**Campesterol**	**54.359**	**1.449**
17	**Stigmasterol**	**54.689**	**1.007**
18	Pregn-5-en-3-ol, 21-bromo-20-methyl-, (3.beta.)-	55.429	4.461

Table 6 shows Sesamin compound (4.5%) that may confer antioxidant and anticancer effects. Other phytochemicals which belongs to Phytosterols were also noticed but to a lesser extent, namely Campesterol (1.5%) and Stigmasterol (1.0%). Phytosterols are structurally similar to cholesterol and are validated by their anti-carcinogenic effects. For example, Campesterol was stated to act as biomarker for cancer prevention [72], and was reported to have potential antiangiogenic action via an inhibition of

endothelial cell proliferation and capillary differentiation [73]. Moreover, Stigmasterol was reported to significantly inhibit tumor promotion in two-stage carcinogenesis in mice [74,75]. Dodecanoic acid isooctyl ester and palmitic acid were the dominant compounds demonstrating 20.9% and 10.58% respectively.

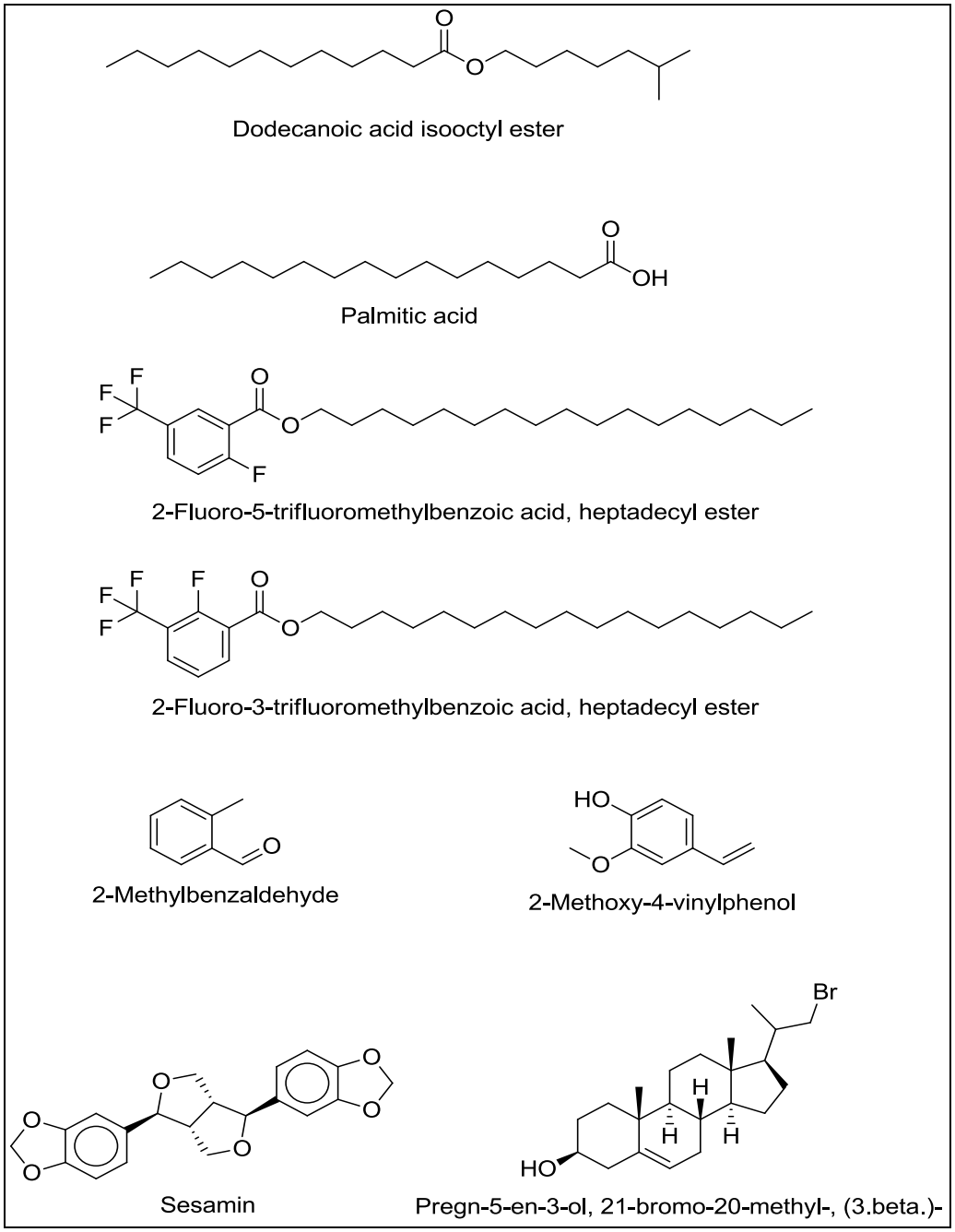


Fig. 11: Chemical structures of the major components in the methanolic *Cuscuta palaestina* extract.

The hexane extract on the other hand was tested for the sake of comparison with the polar methanol extract. It shows less number of compounds mainly hydrocarbons where 8-Hexylpentadecane was the main principle compound (54.65%)

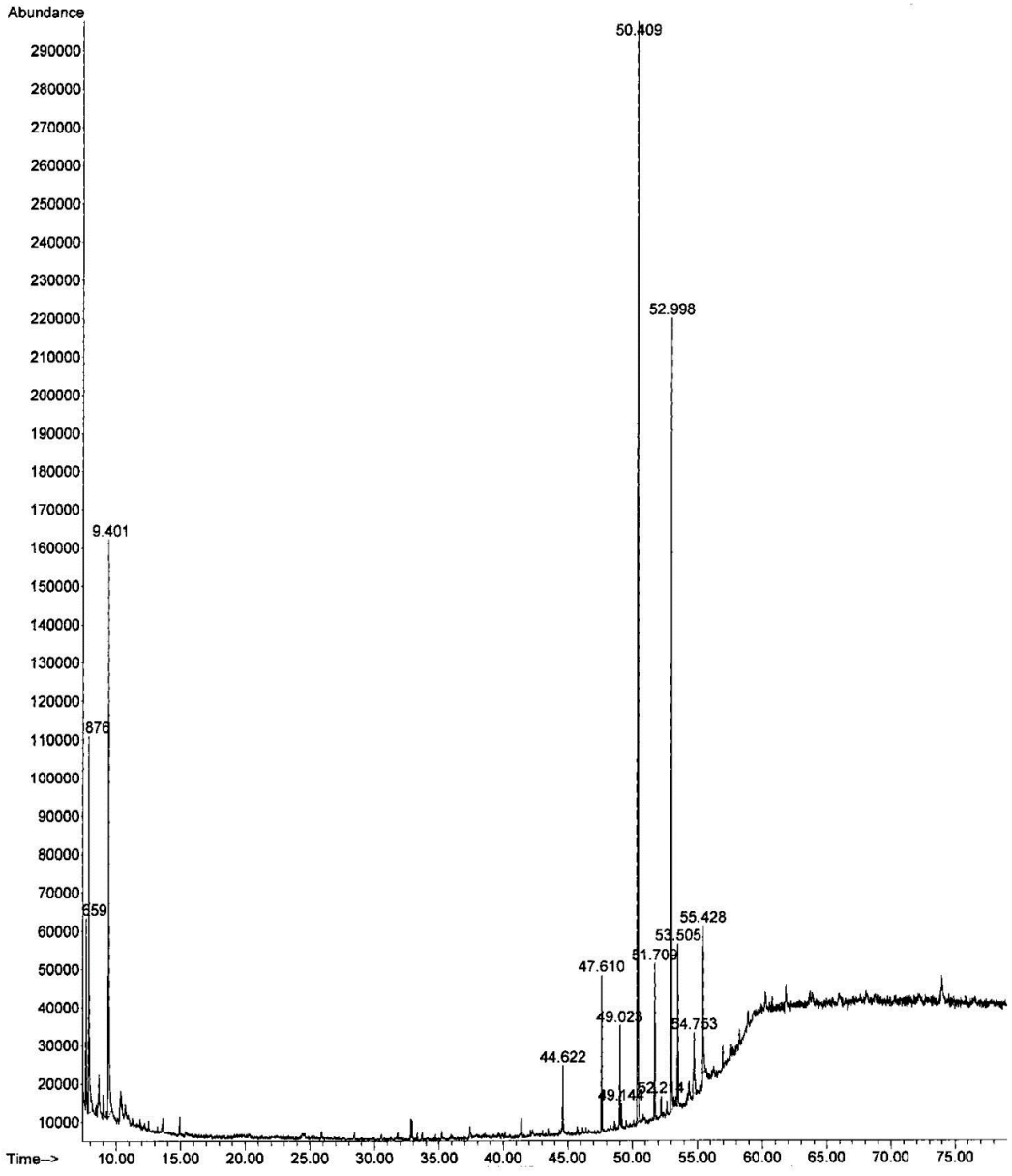


Fig. 10: GC-MS analysis of hexane extract of *Cuscuta palaestina*.

Table 7: Components of *Cuscuta palaestina* hexane extract by GC-MS. Sesamin is bolded and could be the source for the hexane extract anticancer activity.

No.	Compound name	RT (minutes)	% Peak Area
1	Trans-3,4-Dimethyl-2-pentene	9.401	8.418
2	Eicosane	44.622	0.654
3	Heneicosane	47.610	1.782
4	Docosane	49.023	0.98
5	8-Hexylpentadecane	50.409	54.656
6	11-Butyl-docosane	51.709	1.735
7	9-Octylheptadecane	52.998	9.326
8	**Sesamin**	**53.505**	**1.138**
9	2-(1-adamantyl)ethyl 2-phenylacetate	55.428	2.112

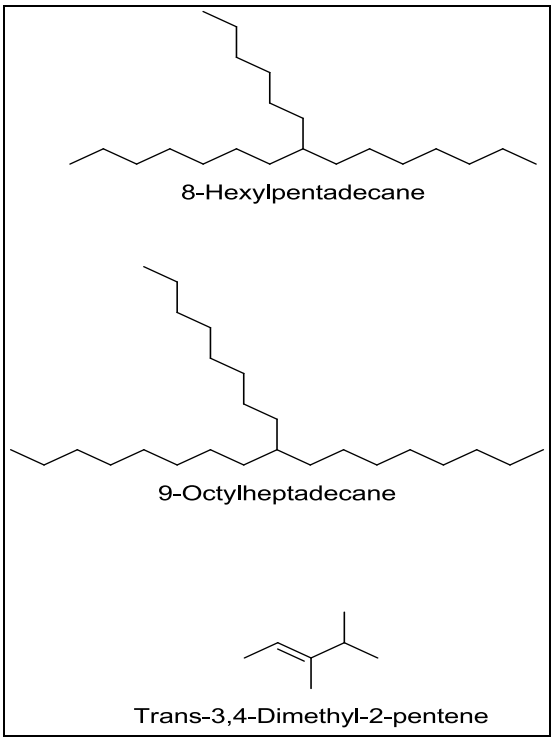


Fig. 11: Chemical structures of the major components in the n-Hexane *Cuscuta palaestina* extract. 8-Hexylpentadecane is the main principle component (54.65%).

Sesamin (Fig. 12) which exists in the oil of Sesame seeds and some other plants, exerts a variety of biological activities, including lipid lowering, antihypertensive, antioxidant and anticancer effects [76,77,78]. As regards to its antitumor effects, Sesamin has already been confirmed to be active against several cancer cell types, including breast cancer [79,80], colon cancer [81], and human lung cancer [82]. Sesamin could be the major source of the anticancer activity of both extracts of *Cuscuta palaestina*, methanolic and hexane, where it constitutes 4.49% and 1.138% respectively. The EC_{50} values that where measured by the MTT assay may support this claim. The values were 71.55 ± 4.75 µg/ml for the methanolic extract and 175.21 ± 3.89 µg/ml for the hexane extract. Show in the Fig. 13, two others Phytosterols namely Campesterol and Stigmasterol could also contribute to the bioactivity of the methanolic extract of *Cuscuta palaestina*. Campesterol and Stigmasterol were not detected in the hexane extract.

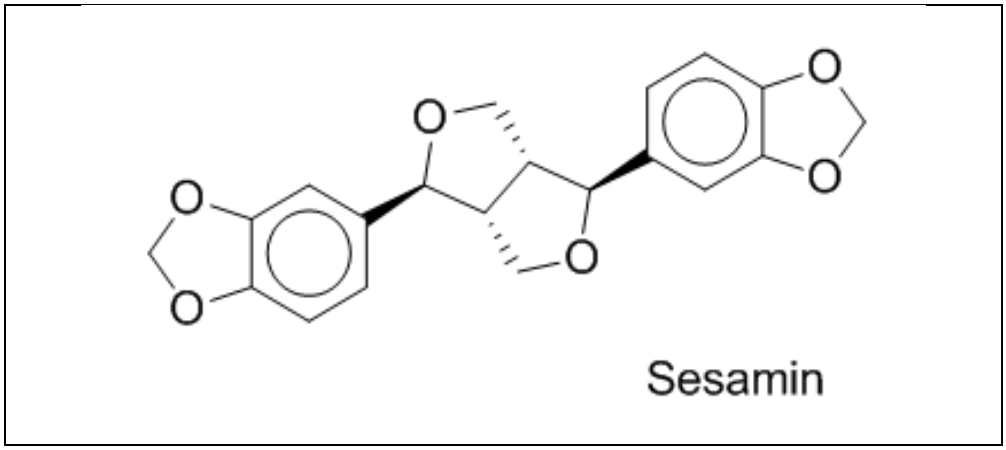


Fig. 12: Chemical structure of Sesamin phytochemical, potentially responsible for anticancer activity in *Cuscuta palaestina* hexane extract and the major source of the methanolic extract activity.

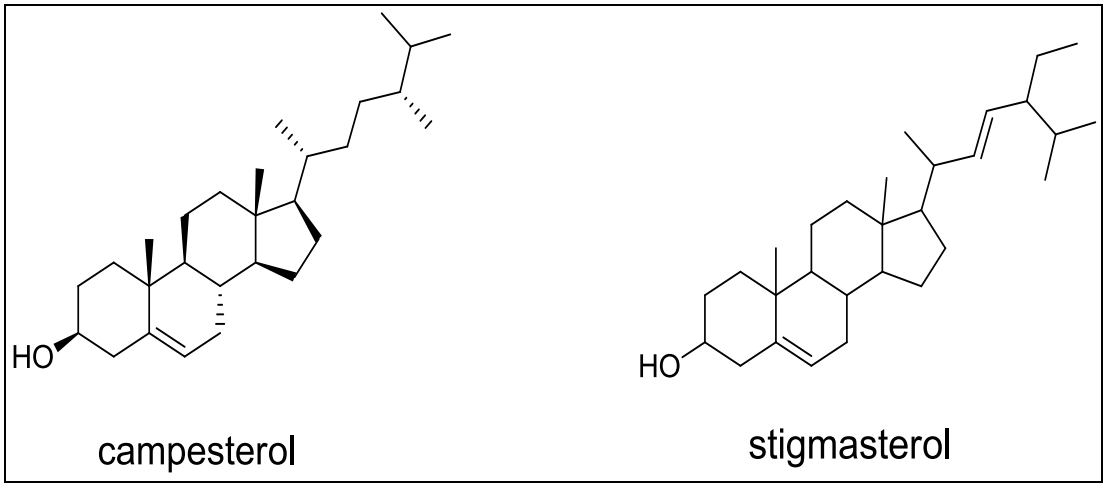


Fig. 13: Chemical structure of Campesterol and Stigmasterol phytochemicals found in the methanolic extract of *Cuscuta palaestina*.

4.1.2. Quantitation of Sesamin in *Cuscuta Palaestina* samples by HPLC-PDA

Fig. 14 shows a typical calibration curve of Sesamin standards including wide range of concentrations. An excellent linearity was achieved as the R^2 value is > 0.999.

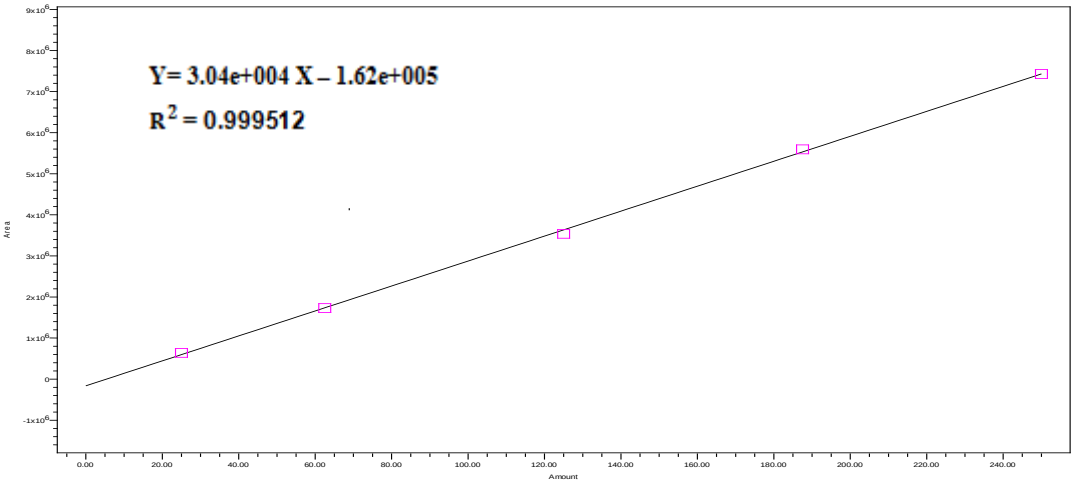


Fig. 14: Calibration curve of Sesamin Compound.

Typical HPLC-PDA Chromatogram of standard Sesamin and its corresponding UV-Vis spectrum which comprises two wavelengths maxima of 235.5 and 286.4 nm is shown in Fig. 15.

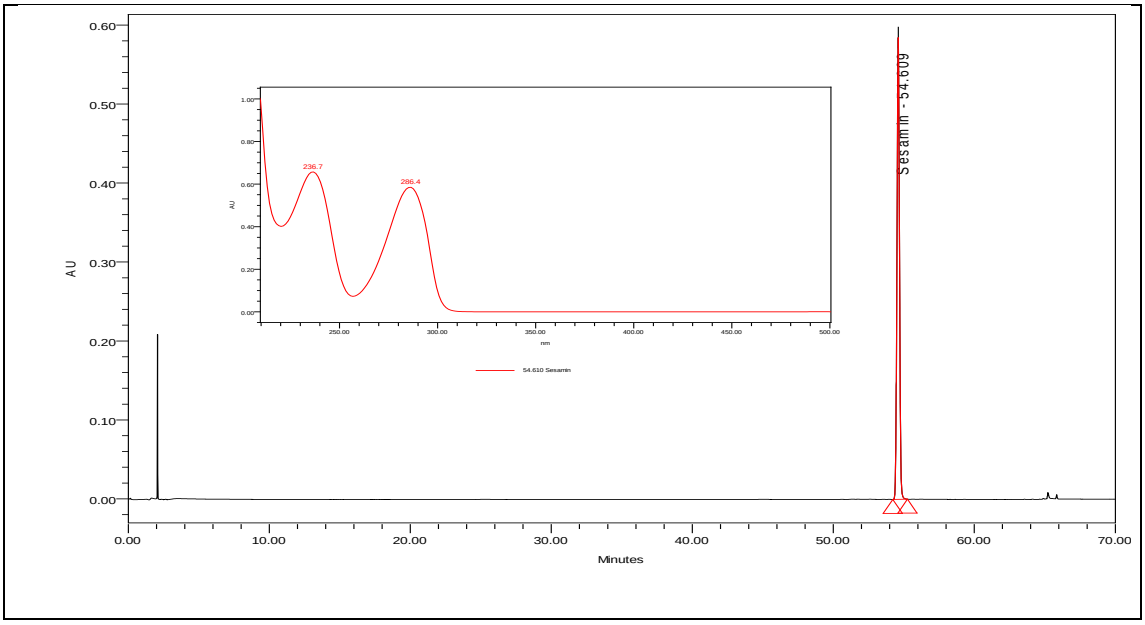


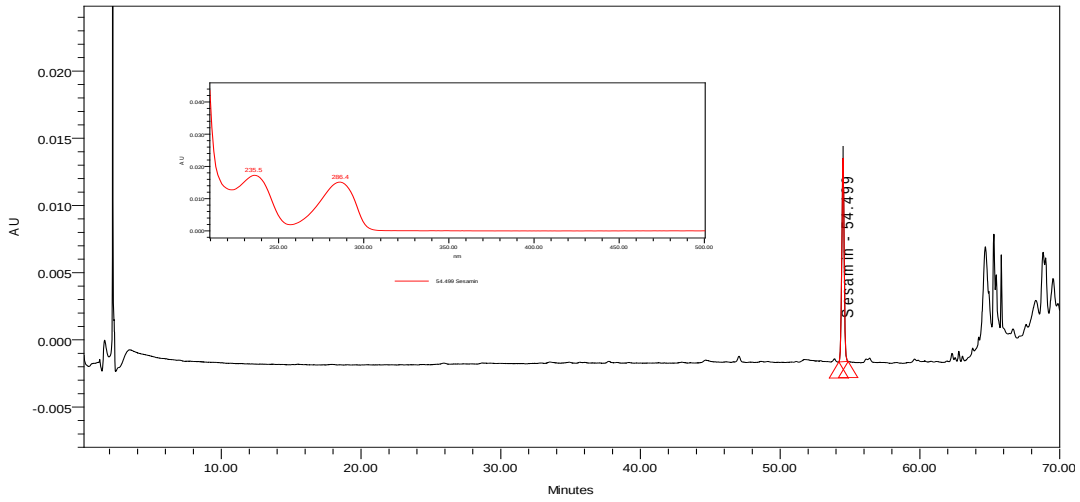
Fig. 15: Typical analytical HPLC-PDA chromatogram of standard Sesamin at concentration of 250 ppm, the UV-Vis spectrum maxima is at λ of 235.5 and 286.4 nm.

Sesamin was eluted at retention time of 54.609 minutes.

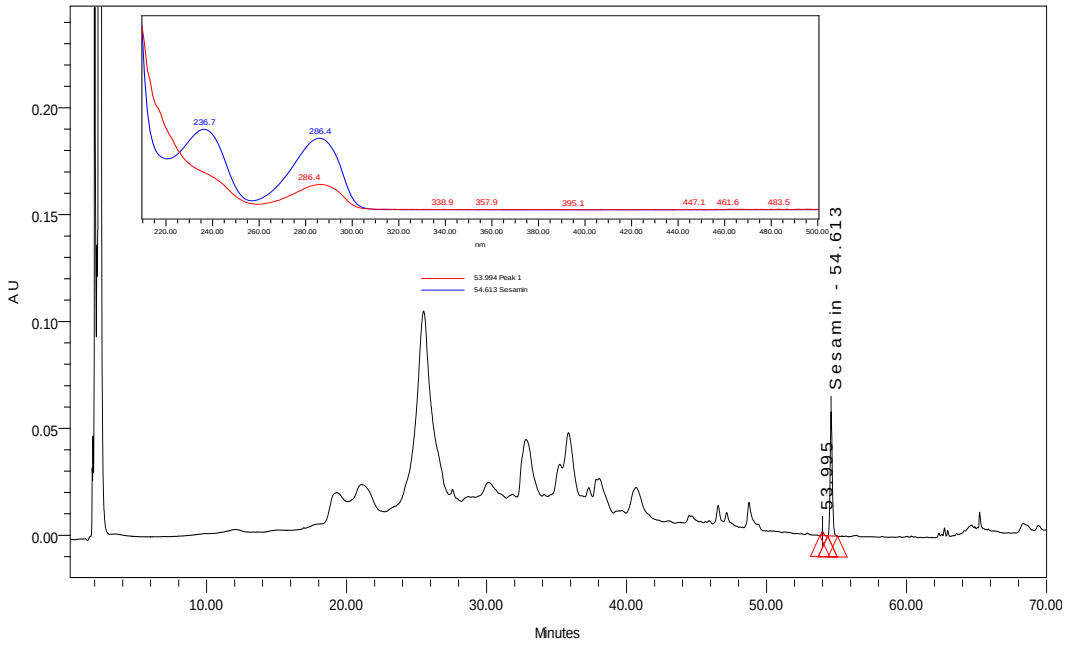
4.1.3. Quantitation of extracted Sesamin using different solvents by HPLC-PDA

Sesamin was extracted from *Cuscuta palaestina* sample using different solvents under the same experimental conditions followed by injection to the HPLC. Retention time and PDA-library which contains sesamin standard UV-Vis spectrum was used to affirm identity and specificity of the extracted sesamin. Fig 16 portrays the chromatographic profile and their corresponding UV-Vis spectrum of the extracted sesamin from hexane, methanol, ethanol and chloroform respectively. Although all the chromatograms were recorded at the maximum wavelength (285 nm) to quantify sesamin, many other peaks were seen preceding and proceeding sesamin in the extracts.

A


AU
0.020
0.015
0.010
0.005
0.000
-0.005
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Minutes
Sesamin - 54.499
235.5
286.4
nm
54.499 Sesamin

B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0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
Minutes
Sesamin - 54.613
53.995
236.7
286.4
286.4
338.9
357.9
395.1
447.1
461.6
483.5
nm
53.994 Peak 1
54.613 Sesamin

C

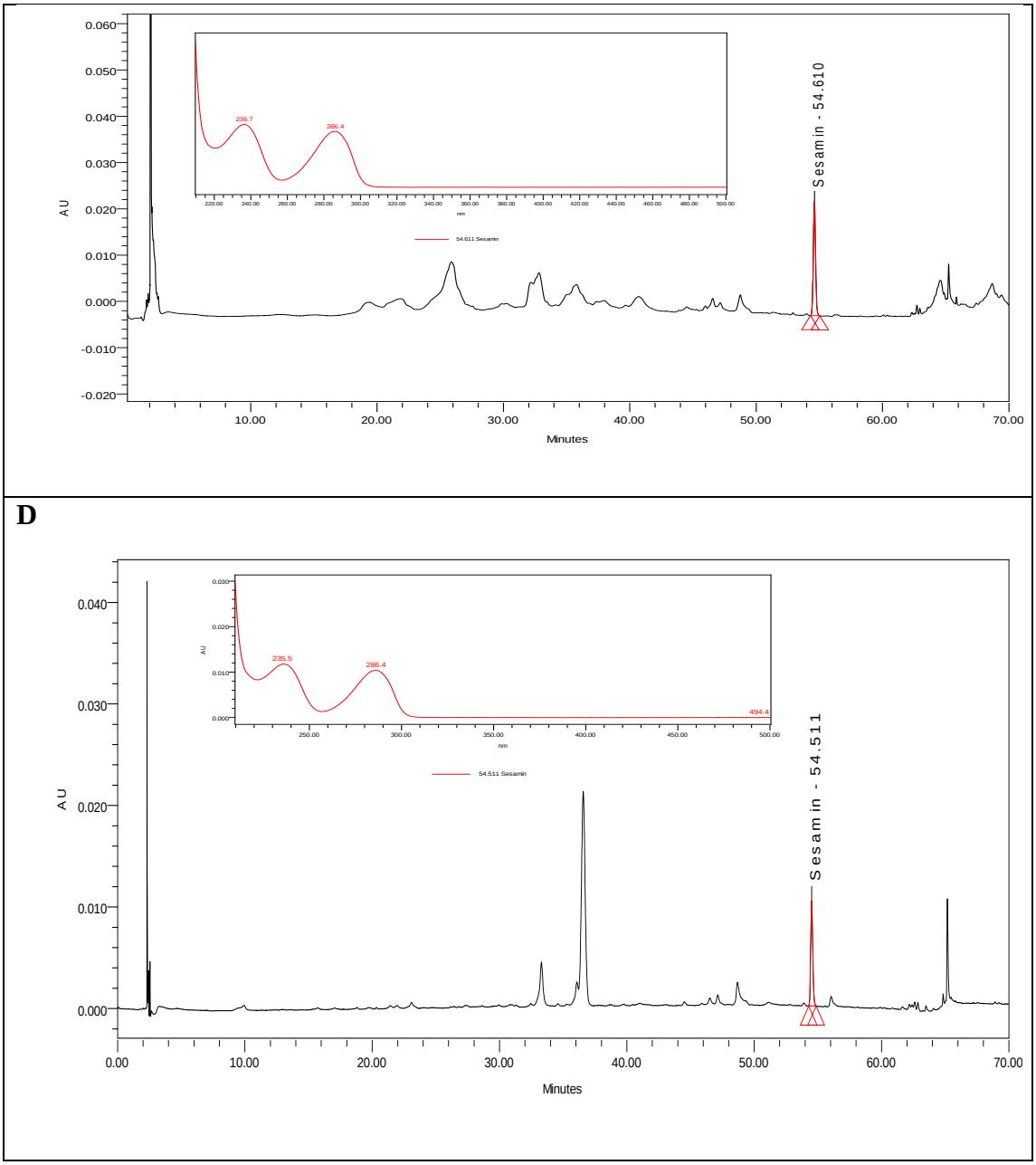


Fig. 16: Typical analytical HPLC-PDA chromatogram of sesamin peak and their relevant UV-Vis spectra (A) hexane extract, (B) methanol extract, (C) ethanol extract, and (D) chloroform extract.

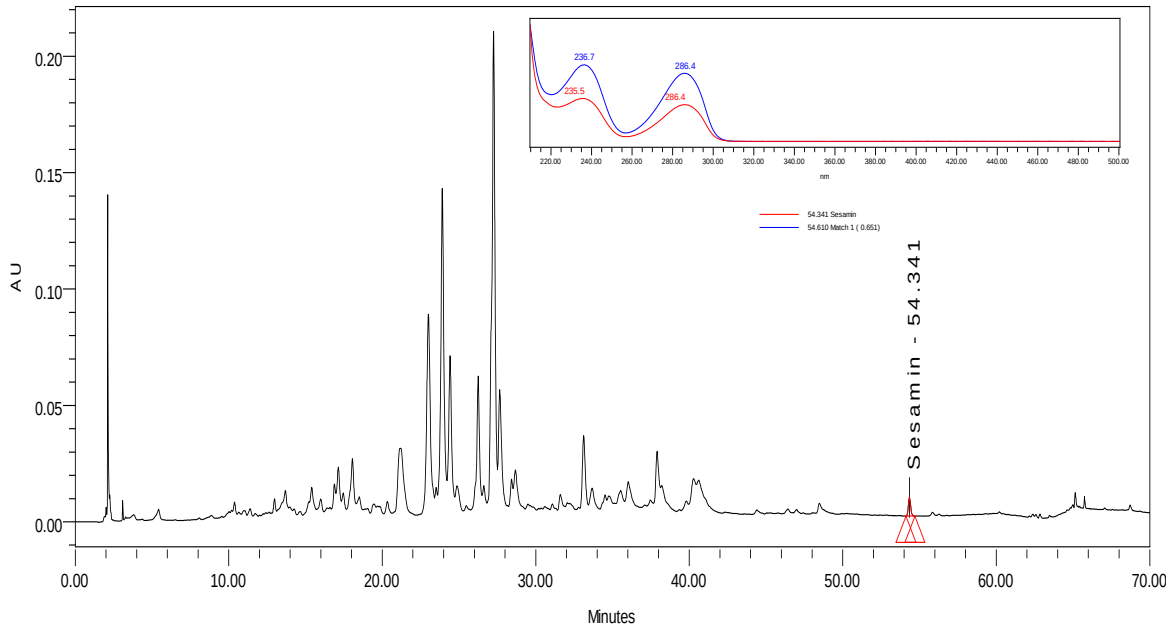
Table 8: Sesamin concentration and % in different solvent.

Extract	Retention time	Area (ppm)	Concentration (ppm)	% Sesamin
A	54.499	175598	6.1	0.68
B	54.613	625860	21.7	0.22
C	54.610	308191	10.7	0.43
D	54.511	116387	9.168	0.53

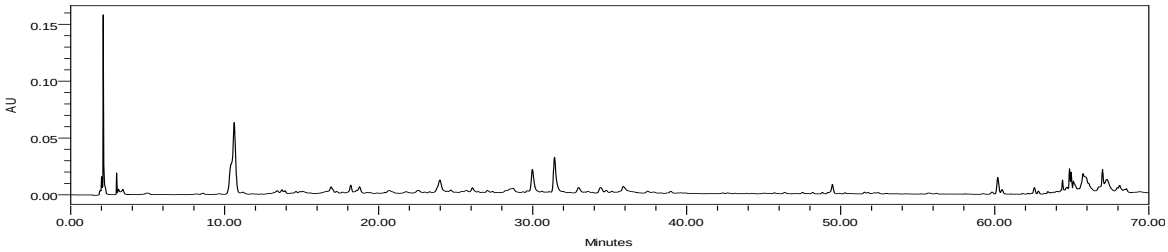
The PDA stored library matching of peak A, B, C, and D extracts with standard Sesamin peak revealed high purity and specificity and therefore possibility of utilizing preparative HPLC for scaling up purposes. Methanol showed higher amount of sesamin (21.7 ppm) in comparison to other solvents and hexane was the lowest (6.1 ppm) while sesamin in the non-polar hexane solvent was the best (table 8).

To prove whether the Sesamin secondary metabolite is endogenous to *Cuscuta palaestina*, or originates merely from the host plant, different samples from the host plant alone along with *Cuscuta palaestina* with the host plant were extracted and the sesamin concentration was calculated as shown in Fig. 17.

A



B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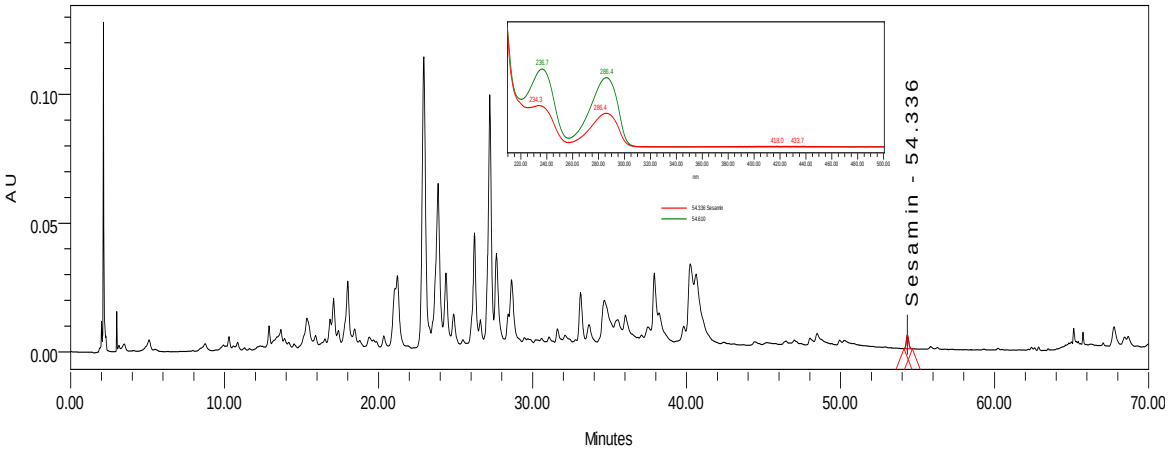
Minutes

C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Sesamin - 54.336
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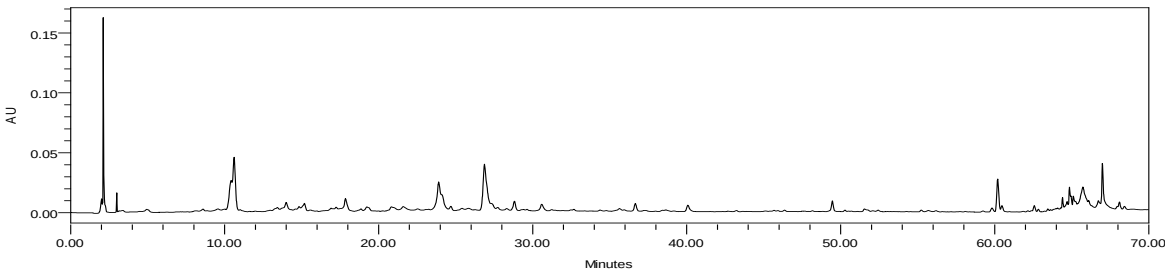
Minutes

D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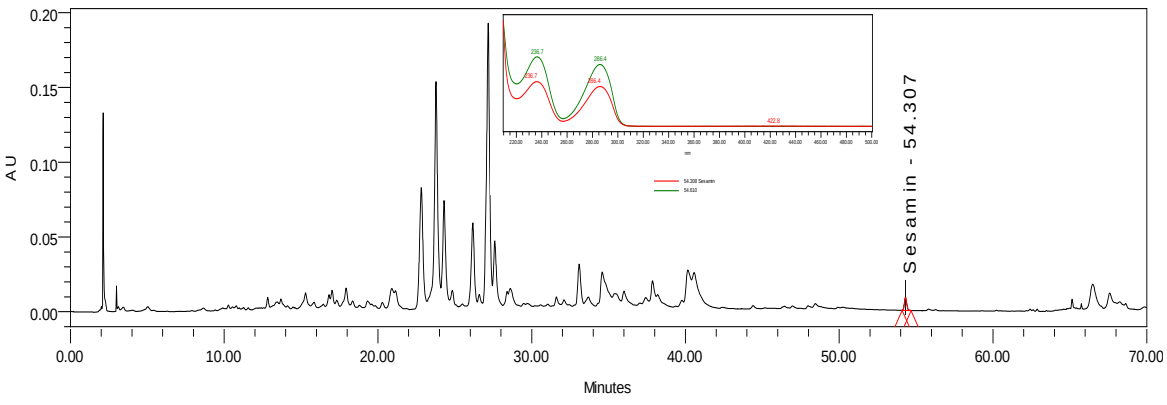
E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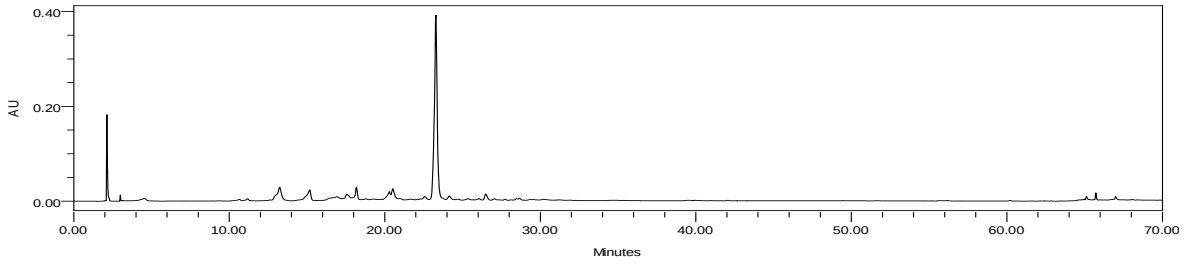
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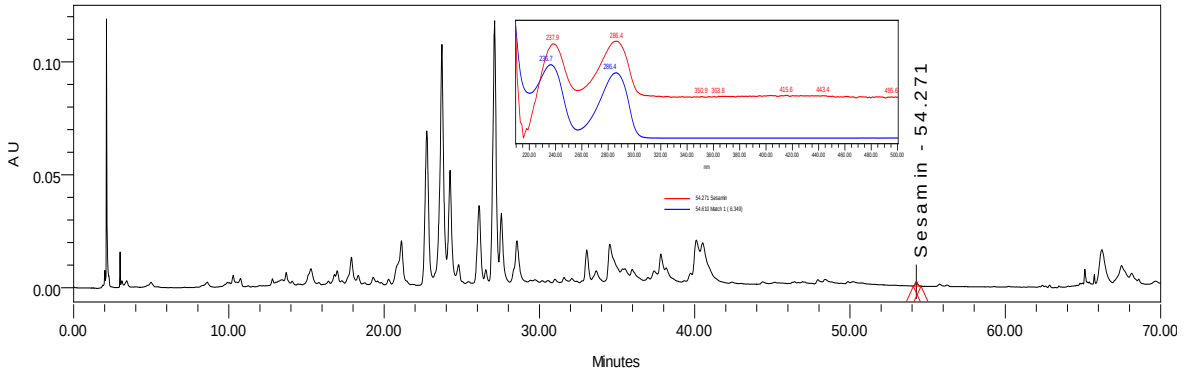
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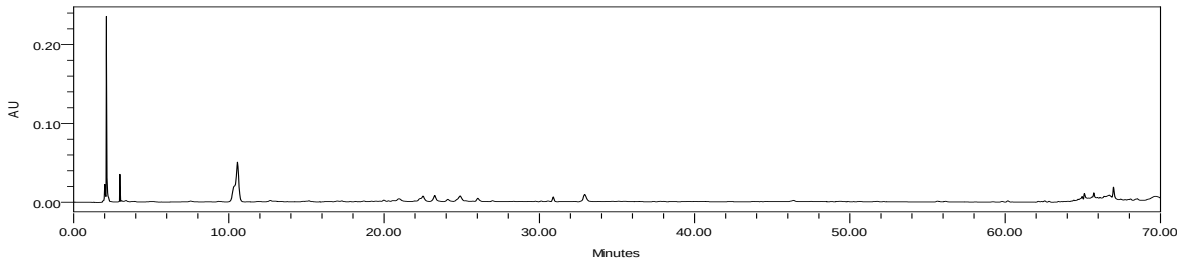
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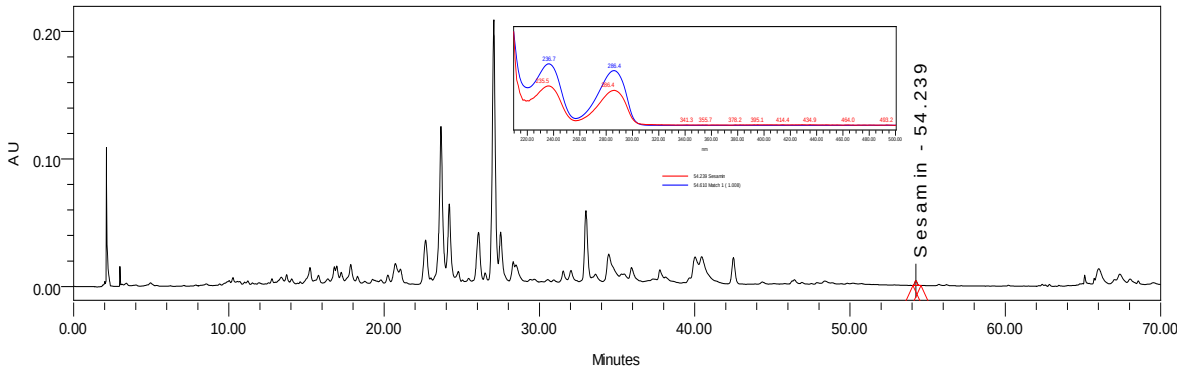
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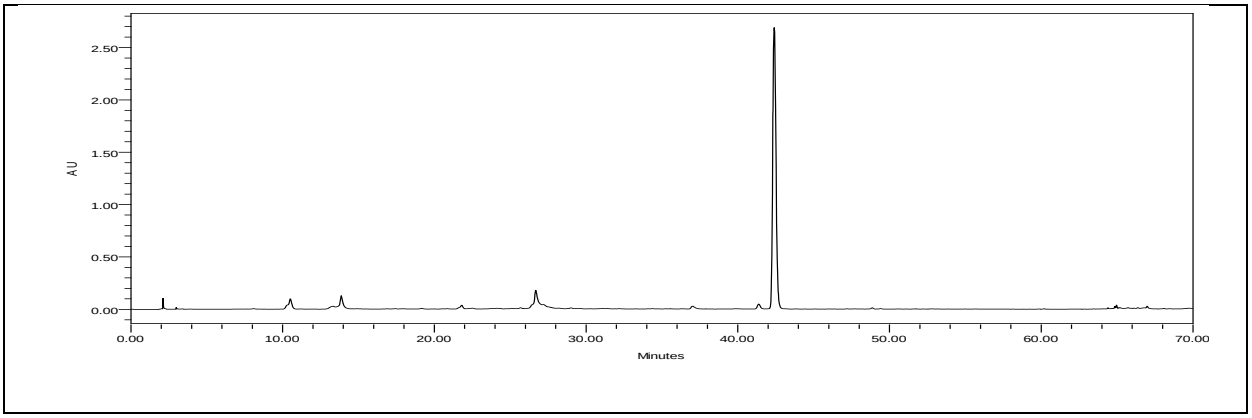


Fig. 17: (A) The chromatogram and UV-Vis spectra of extracted *Cuscuta palaestina + Malva sylvestris*, (B) The chromatogram *Malva sylvestris*, (C) The chromatogram and spectrum of *Cuscuta palaestina + Cichorium Intybus* (D) The chromatogram of *Cichorium Intybus*, (E) The chromatogram and spectrum of *Cuscuta palaestina + Prosopis farcta*, (F) The chromatogram of *Prosopis farcta*, (G) The chromatogram and spectrum of *Cuscuta palaestina + Portulaca oleracea* (H) The chromatogram of *Portulaca oleracea*, (I) The chromatogram and spectrum of *Cuscuta palaestina + X* (unknown plant), (J) The chromatogram of *X* (unknown plant).

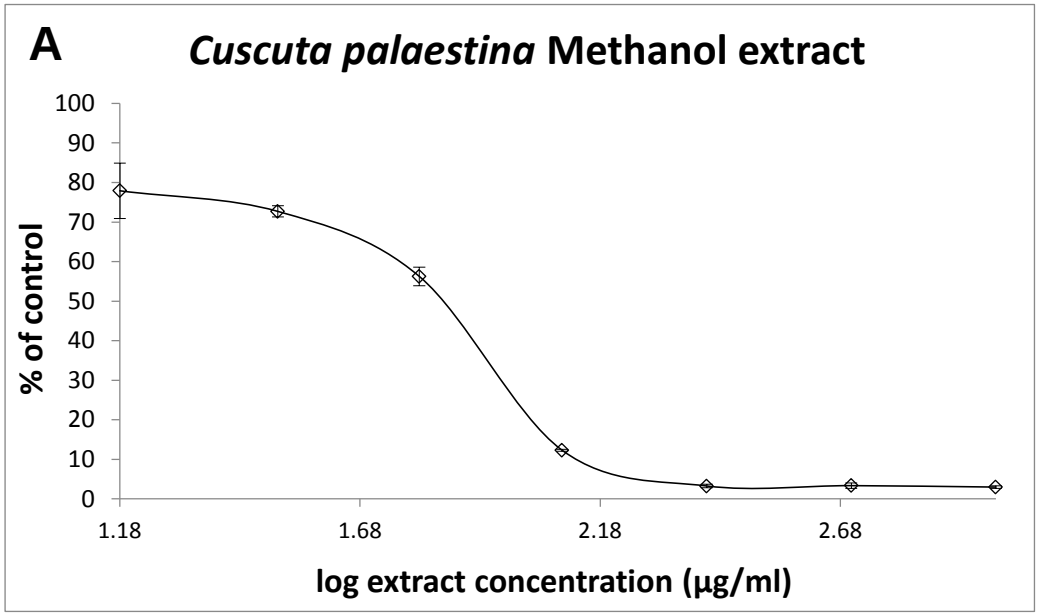
Table 9 shows Sesamin concentration in methanolic extract of *Cuscuta Palaestina* that are parasite to plants (A and C and E and G and I. Since Sesamin peaks were not seen in the chromatograms of the host plants, therefore, it was concluded that Sesamin is endogenous to *Cuscuta Palaestina*.

Table 9: Sesamin concentration in *Cuscuta Palaestina* that are parasite on plants.

Sample	Retention time (t_R)	Area	% Area	Concentration (ppm)
A	54.341	86256	100.00	8.174
C	54.336	59728	100.00	7.301
E	54.307	94565	100.00	8.448
G	54.271	21222	100.00	6.033
I	54.239	40460	100.00	6.666

4.1.4. Anticancer activity of *Cuscuta palaestina* methanolic and hexane extracts

Cytotoxicity experiments were conducted on *Cuscuta palaestina* methanolic and hexane extracts to disclose the effectiveness of *Cuscuta palaestina* in cessation of tumor growth in cancer treatment. Through the *in vitro* bioactivity assay, the human colon cancer cell line (HCT-116) was exposed to *Cuscuta palaestina* methanol and hexane extracts (0–1000 µg/mL) for 24 h, and cytotoxicity on cancer cell line was determined by using the MTT and the LDH leakage assays. The EC_{50} values obtained by the MTT assay was 71.55 ± 4.75 µg/ml for the Methanolic extract and 175.21 ± 3.89 µg/ml for the Hexane extract. Figures 18 and 19 summarizes results for MTT and LDH leakage assays for the *Cuscuta palaestina* methanol and hexane extract respectively. The analysis of the results was carried out using Student's *t*-test for unpaired two-tailed comparisons; p-values less than 0.05 were considered significant. The obtained results indicate that tested *Cuscuta palaestina*, especially the methanol extract has an anti-proliferative activity against human Colon carcinoma cell line with significant effect.



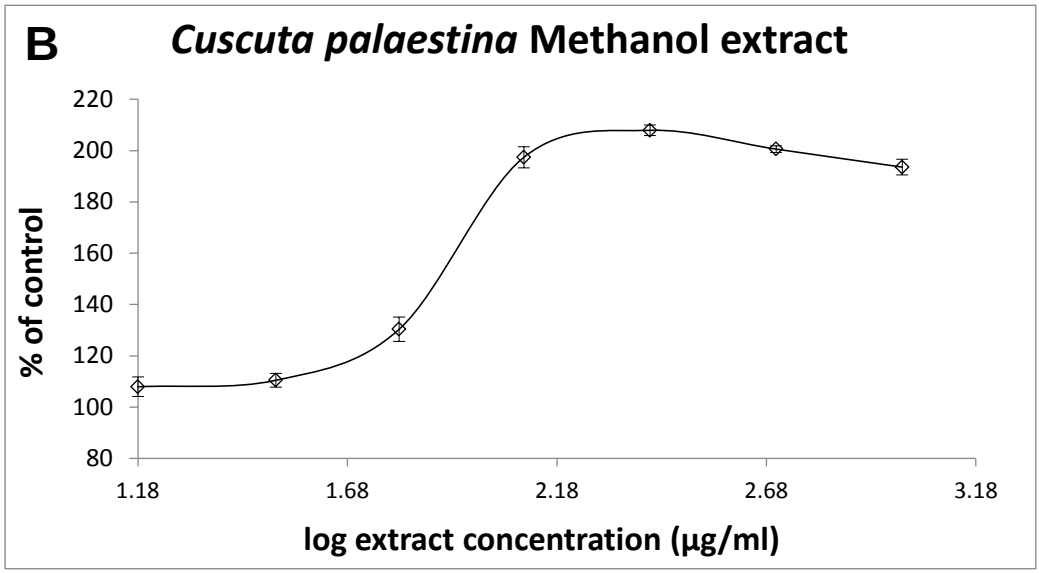
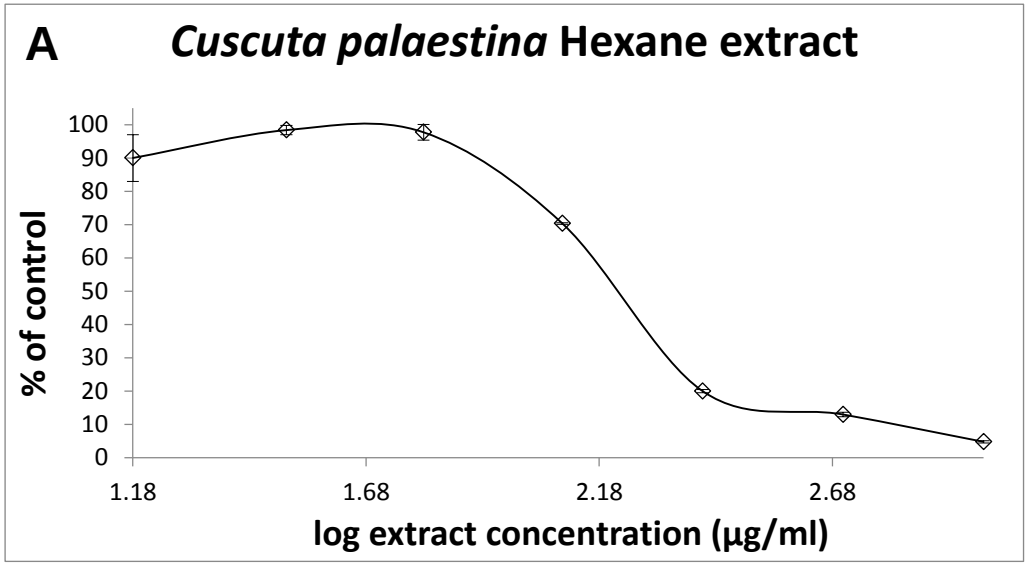


Fig. 18: MTT (A), LDH leakage (B) assays in HCT-116 (Colon carcinoma, human) after exposure to *Cuscuta palaestina* methanolic extract for 24 h. Data in A and B presented as percentage of control (*n=3*, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.



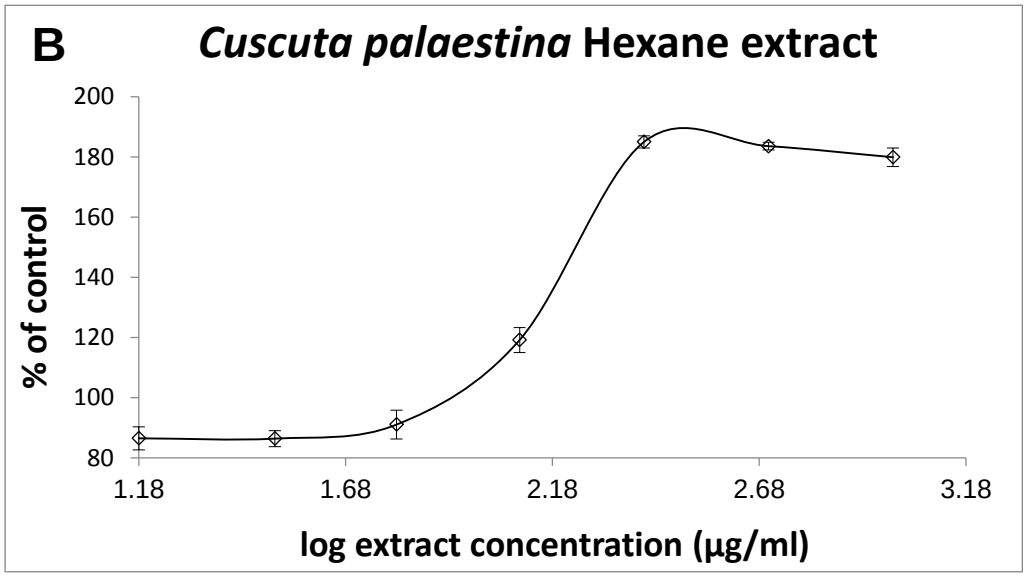


Fig. 19: MTT (A), LDH leakage (B) assays in HCT-116 (Colon carcinoma, human) after exposure to *Cuscuta palaestina* Hexane extract for 24 h. Data in A and B presented as percentage of control (*n=3*, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

4.2. *Gundelia tournefortii* plant

4.2.1. GC-MS phytochemical analysis of *Gundelia tournefortii*

The phytochemical screening using GC-MS in the electron impact mode (EI) revealed 70 compounds in *Gundelia tournefortii* methanolic and hexane extracts, 64 of which

are detected for the first time in *Gundelia tournefortii*. Six anticancer famous components, namely, Stigmasterol, β-Sitosterol, Palmitic acid, Linoleic acid, α-Linolenic acid and Stearic acid were previously reported in the literature [83]. The result of GC-MS analysis without derivatization of *Gundelia tournefortii* revealed 14 major components in the hexane extracts as shown in Fig.20, and Table 10, and Fig.21.

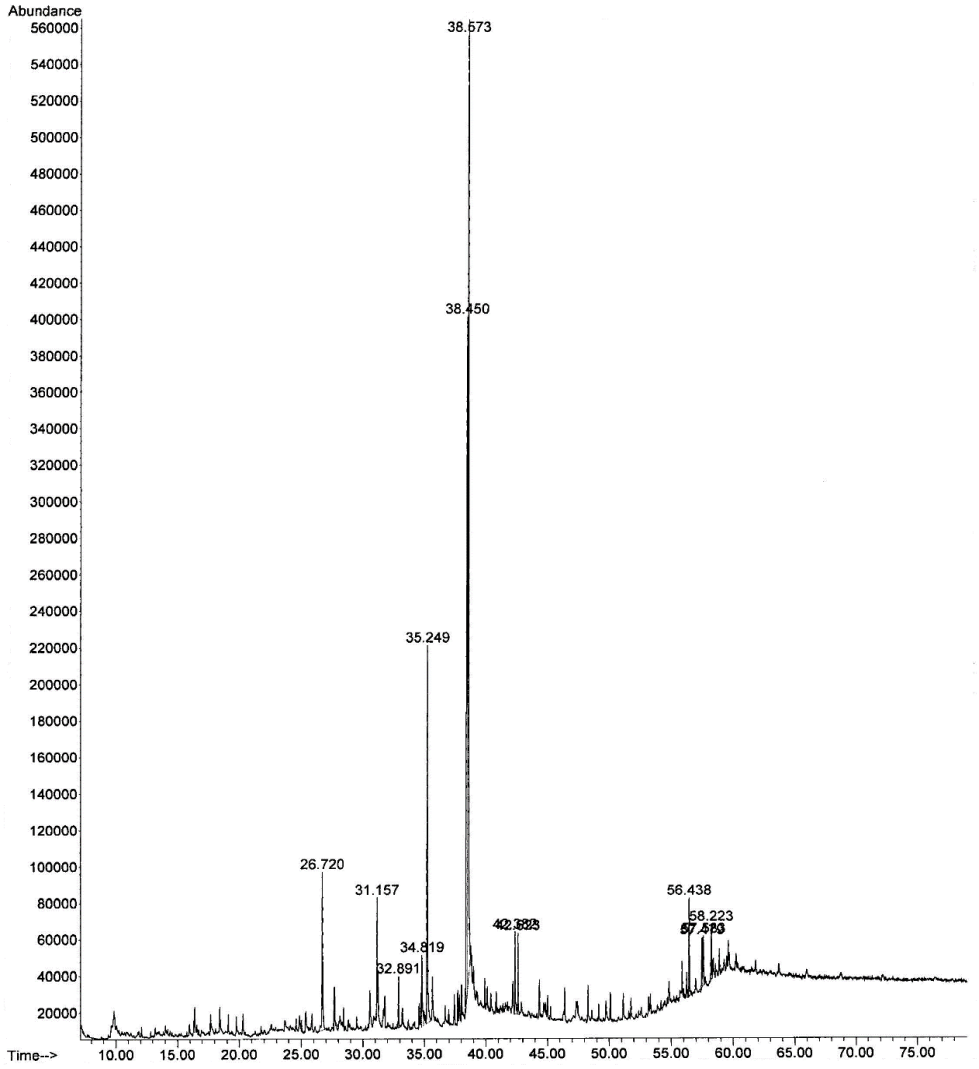


Fig. 20: Total ion chromatogram (TIC) of *Gundelia tournefortii* hexane without derivatization extract.

Table 10: Phytochemicals of *Gundelia tournefortii* from hexane extract by GC-MS.

No	Component name	RT (minutes)	% Peak area
1	Methyl palmitate	35.91	1.89
2	Methyl linoleate	38.94	1.27
3	Ethyl oleate	39.06	1.65
4	NI*	50.39	1.96
5	Heptadecane	53.01	4.21
6	**Stigmasterol**	54.69	5.75
7	**β-Sitosterol**	55.44	5.09
8	α-Amyrin	55.89	5.76
9	**Lupeol**	56.45	9.17
10	Olean-12-en-3-yl acetate	56.95	9.58
11	Thunbergol	57.49	24.41
12	Hop-22(29)-en-3.beta.-ol	57.59	11.31
13	NI	58.45	6.58
14	A'-Neogammacer-22(29)-en-3-ol, acetate, (3.beta.,21.beta.)-	58.58	8.341

*NI: not identified

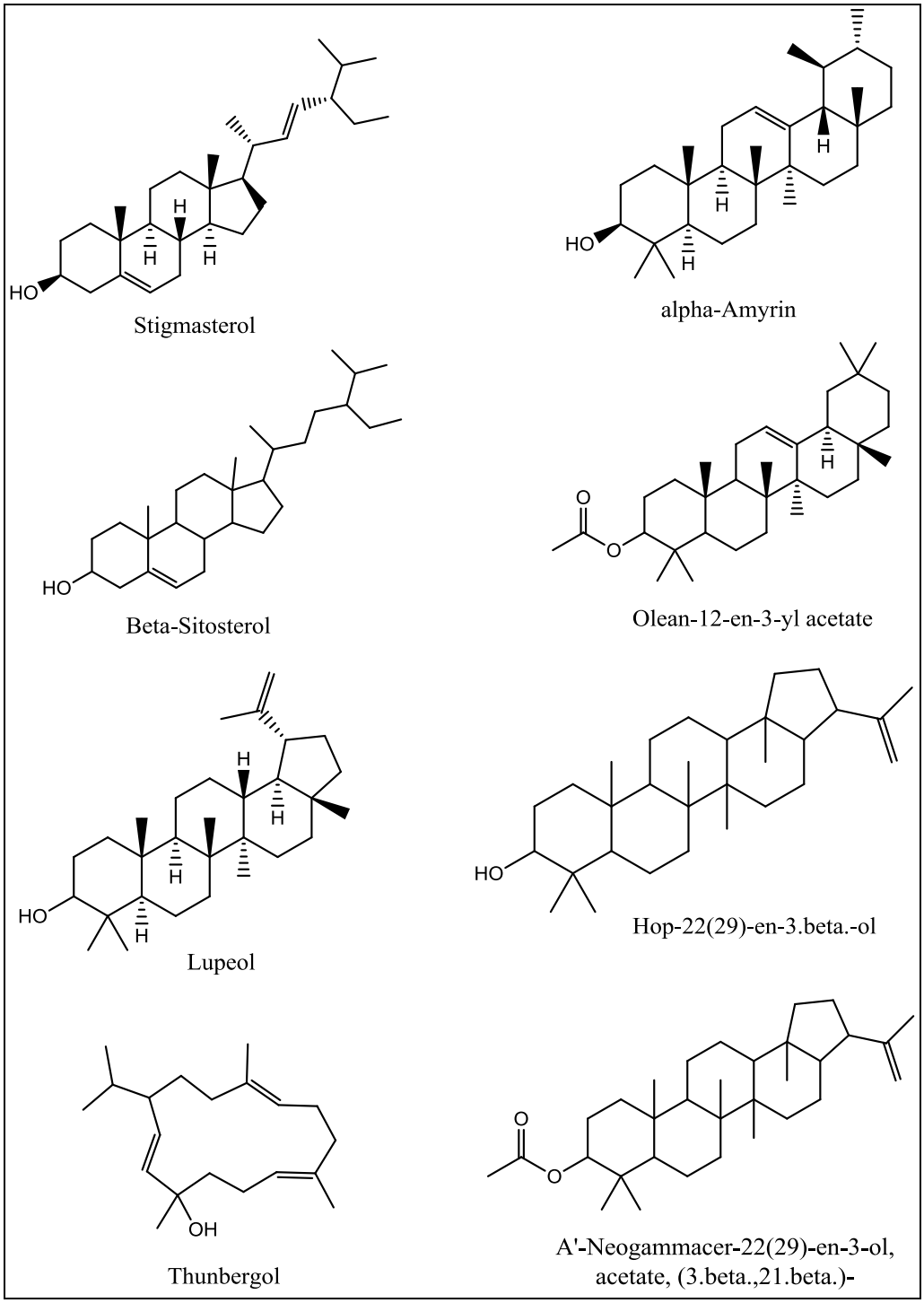


Fig. 21: Chemical structure of major components in *Gundelia tournefortii* hexane without derivatized extract.

The result of GC-MS analysis without derivatization of *Gundelia tournefortii* revealed 13 major components in the methanol extracts as shown in Fig. 22, and Table 11, and Fig. 23.

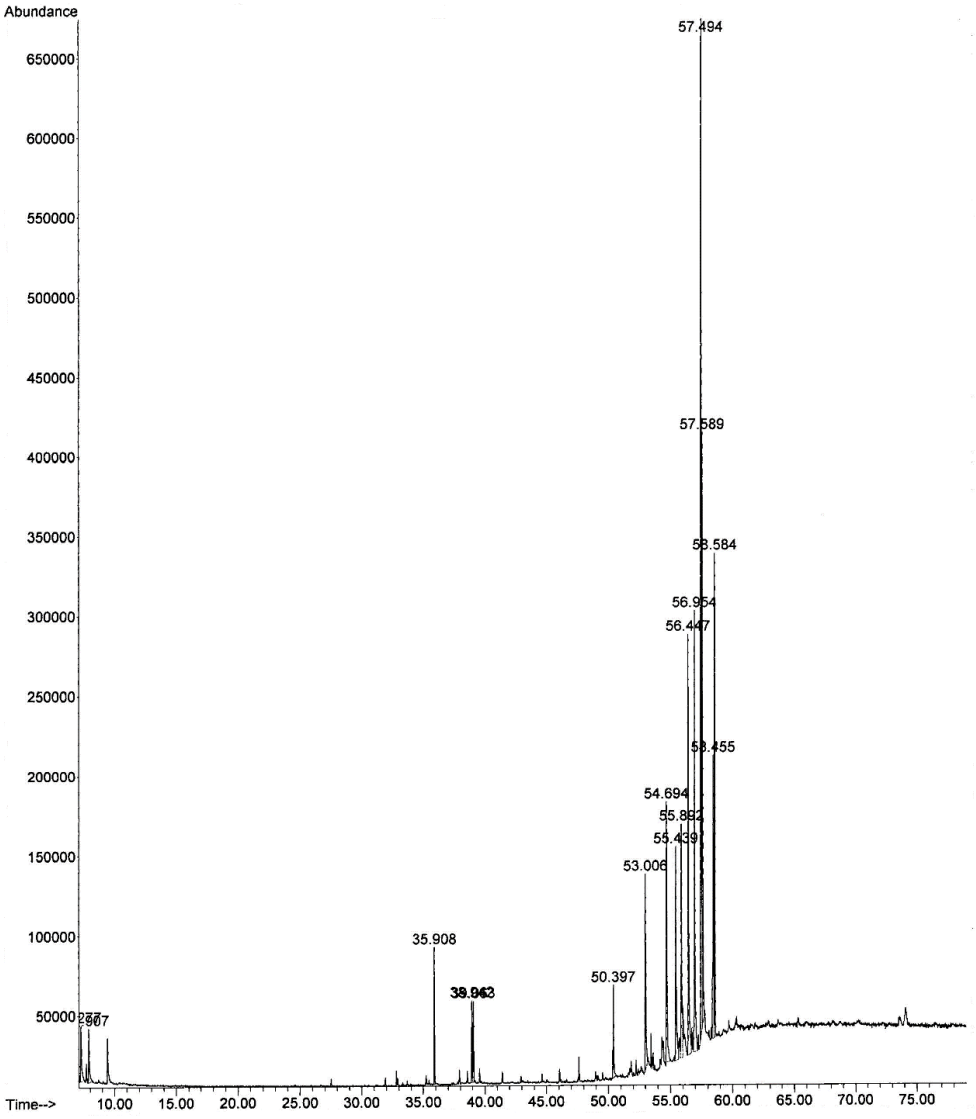


Fig. 22: Total ion chromatogram (TIC) of *Gundelia tournefortii* methanol without derivatization extract.

Table 11. Phytochemicals of *Gundelia tournefortii* from methanol extract by GC-MS.

No	**Component name**	**RT (minutes)**	**% Peak area**
1	Dodecanoic acid	26.72	5.16
2	Tetradecanoic acid	31.16	1.58
3	6,10,14-Trimethylpentadecan-2-one	32.89	1.18
4	(Z)-11-hexadecenoic acid	34.82	2.41
5	Palmitic acid	35.25	11.70
6	Linoleic acid	38.45	26.88
7	(9Z)-9,17-Octadecadienal	38.57	39.28
8	**Artemisinin**	42.38	2.39
9	Matricarin	42.62	1.97
10	Hop-22(29)-en-3.beta.ol	56.44	2.81
11	NI*	57.48	1.53
12	NI	57.58	1.59
13	**Gitoxigenin**	58.22	1.51

*NI: not identified

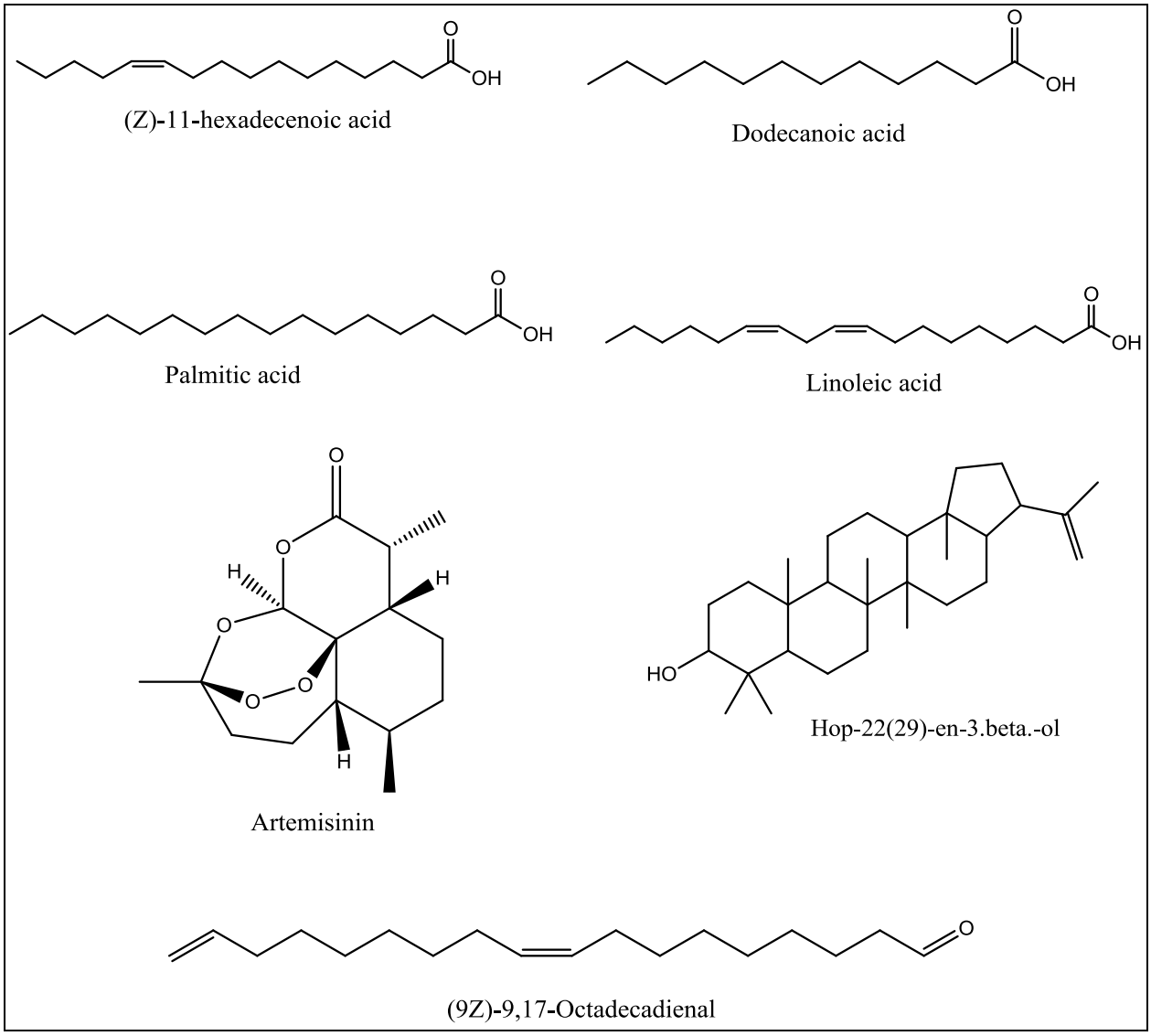


Fig. 23: Chemical structure of major components in *Gundelia tournefortii* methanol without derivatization extract.

Although a satisfactory resolution, selectivity and elution time were obtained on the capillary GC HP-5 column, however, the derivatization of the secondary metabolites of *Gundelia tournefortii* seemed to be helpful due to presence of polar phytochemicals. Typically, upon deivatization with BSTFA reagent, more volatile and stable compounds were generated with amendable properties to GC-MS analysis. We noticed a doubled number of sharper peaks culminated, namely 26 peaks in the derivatized hexane extracts (Fig. 24, and table 12, and Fig. 25), and methanol extracts (Fig. 26, table 13, and Fig. 27).

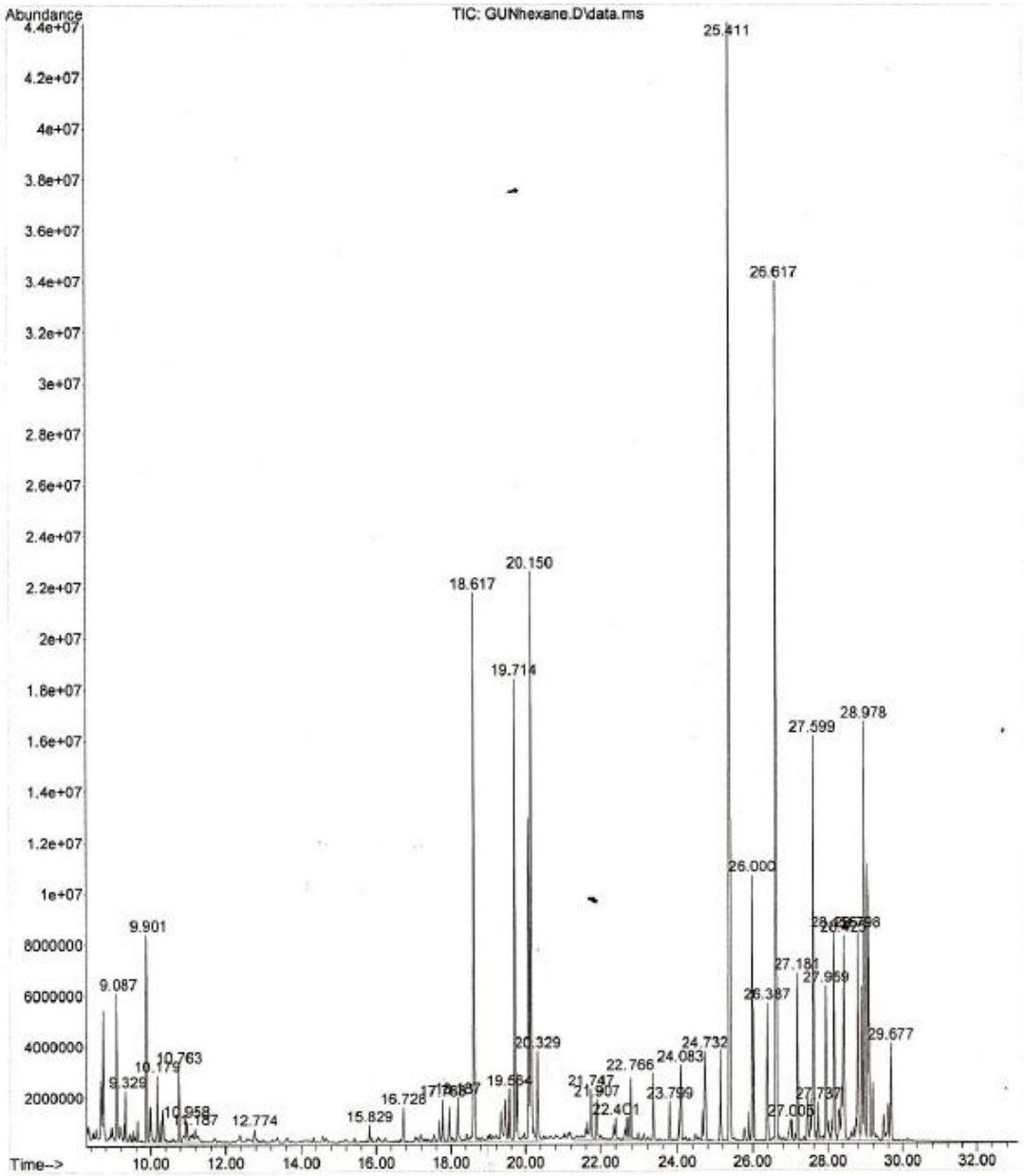


Fig. 24: Total ion chromatogram (TIC) of *Gundelia tournefortii* silylated BSTFA hexane extract.

Table 12: Phytochemicals of *Gundelia tournefortii* from hexane silylated BSTFA extract verified by GC-MS.

No.	Component name	RT (minutes)	% Peak area
1	3-Methyl-2-butanol	9.33	0.85
2	1-Methylphenanthrene	9.907	3.14
3	4-Methylpent-1-ene-2,4-diol	10.17	1.21
4	D-(-)-Lactic acid	11.18	0.64
5	Propanoic acid	12.77	0.34
6	Tetradecanoic acid	16.726	0.51
7	Cetanol	17.78	1.48
8	Ethyl icosanoate	18.18	0.58
9	Tetrandecanioc acid	18.613	5.06
10	Octadecan-1-ol	19.556	2.2
11	(E)-3,7,11,15-tetramethylhexadec-2-en-1-ol.	19.714	4.68
12	α-Linolenic acid	20.152	9.38
13	Stearic acid	20.317	0.95
14	Oleamide	21.74	1.37
15	Eicosanoic acid	21.906	0.71
16	5-Octadecene	22.402	0.51
17	Di-n-Octylphtalate	22.76	1.99
18	Tetracosan-1-ol	24.09	1.09
19	Hexacosan-1-ol	25.414	15.07
20	Heptacosane	26.39	1.05
21	Octacosan-1-ol	26.623	9.15
22	Fructose oxime	27.00	0.49
23	**Stigmasterol**	27.599	4.73
24	**β-sitosterol**	27.95	1.91
25	12-Oleanen-3-yl acetate	28.791	2.69
26	Hop-22(29)-en-3beta-ol	29.67	1.97

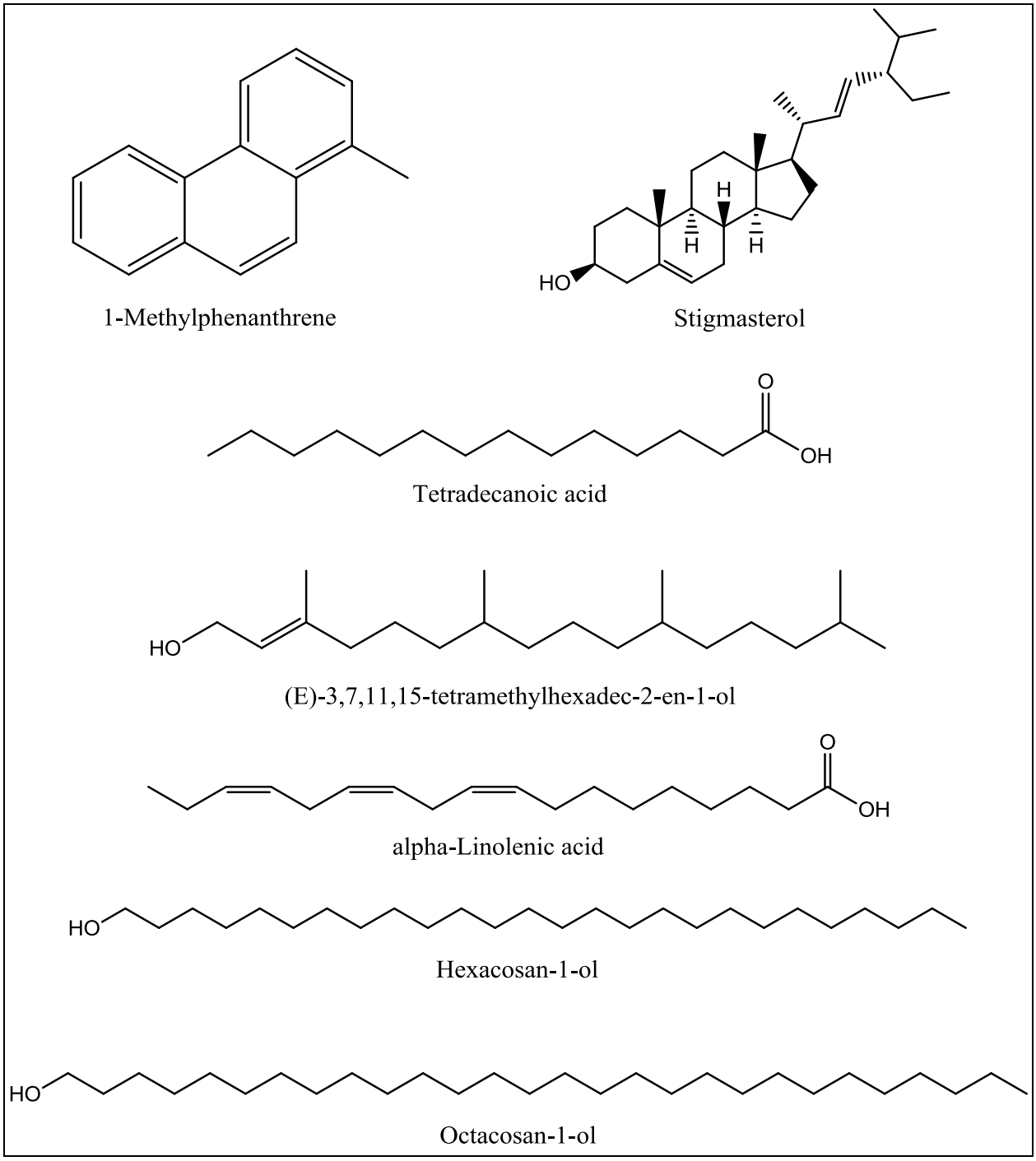


Fig. 25: Chemical structure of major components in *Gundelia tournefortii* silylated BSTFA hexane extract.

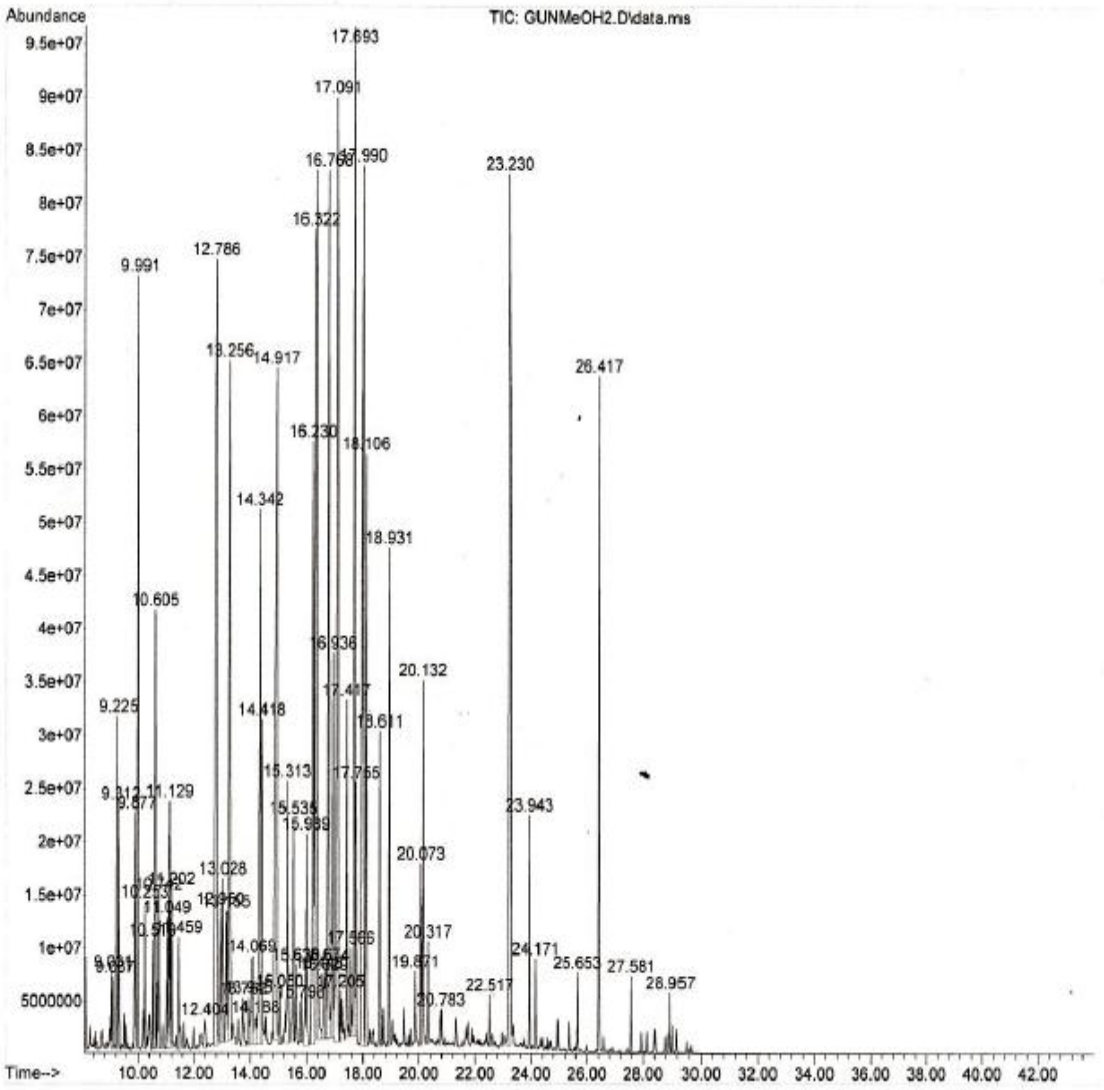


Fig. 26: Total ion chromatogram (TIC) of *Gundelia tournefortii* silylated BSTFA methanol extract.

Table 13: Phytochemicals of *Gundelia tournefortii* from methanol silylated BSTFA extract verified by GC-MS.

No	Compound name	RT (minute)	% Peak area
1	DL-Valine	9.2	0.99
2	Aminoethanol	9.87	0.93
3	Glycerol	9.99	3.59
4	L-Isoleucine	10.255	0.45
5	Succinic acid	10.6	2.2
6	Glyceric acid	10.75	0.68
7	Fumaric acid	11.05	0.46
8	DL-Serine	11.15	0.95
9	9-Piperidine carboxylic acid	11.198	0.58
10	Threonine	11.46	0.52
11	Malic acid	12.79	7.58
12	L-Aspartic acid	13.151	0.56
13	4-Amino butanic acid	13.27	4.93
14	Asparagin	14.94	6.15
15	Xylitol	15.05	0.28
16	Arabitol	15.32	1.12
17	D-Ribofuranose	16.23	3.96
18	D-Fructose	16.33	5.47
19	D-Galactofuranose	16.625	0.45
20	Lyxose	17.1	6.88
21	Sorbitol	17.42	1.36
22	Inositol	17.7	5.09
23	Glucopyranose	18.00	5.01
24	D-Gluconic acid	18.15	2.61
25	Palmitic acid	18.61	0.95
26	Linoleic acid	20.069	0.66
27	L-Tryptophan	20.135	1.22
28	Methyl α-D-glucopyranoside	23.23	7.04
29	D-Xylonic acid	24.17	0.33
30	**Stigmasterol**	27.58	0.24

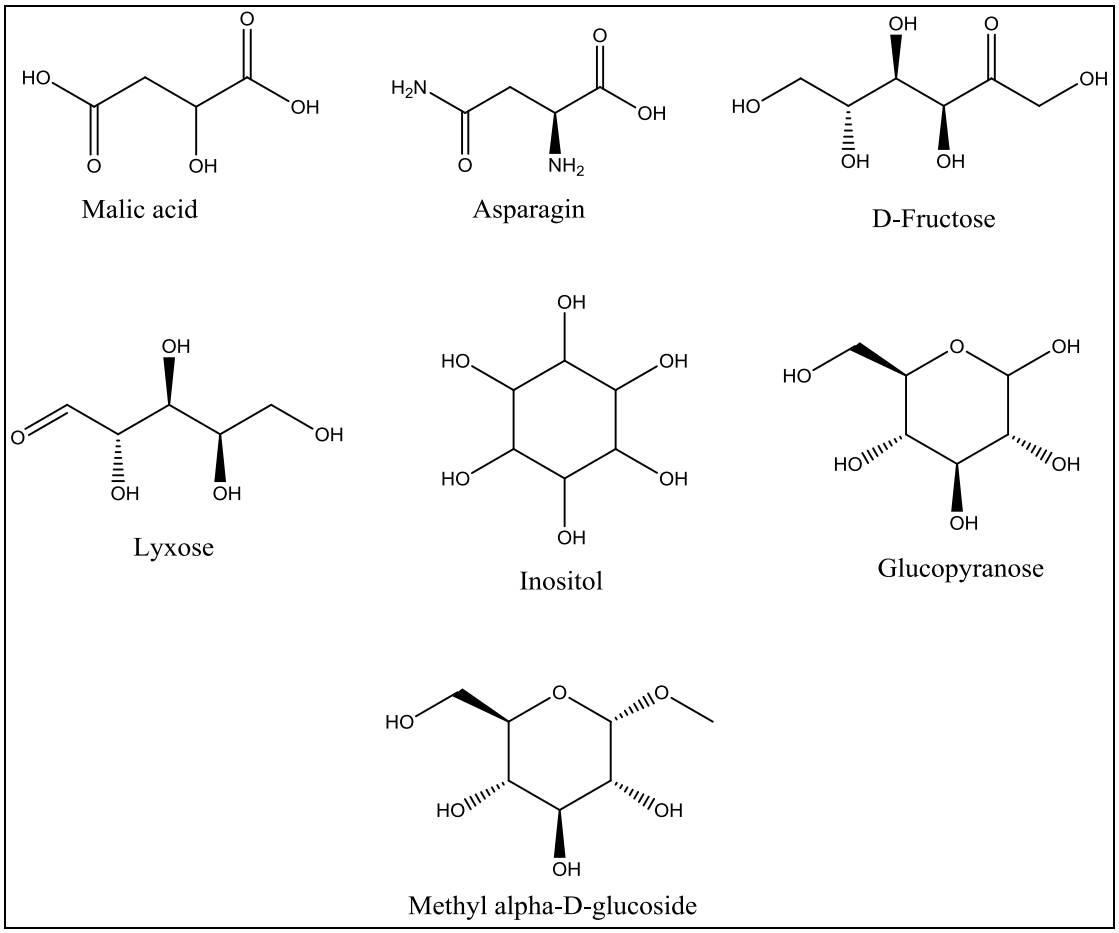


Fig. 27: Chemical structure of major components in *Gundelia tournefortii* methanol silylated BSTFA extract.

Sitosterol and stigmasterol were seen after derivatization in the hexane extract (Table 15, Fig. 28) while stigmasterol alone was seen in the methanolic derivatized extract (Table 16). Although there were few active compounds that were seen in both pre- and post-derivatization extracts, the more effective phytochemicals were more in the underivatized batch.

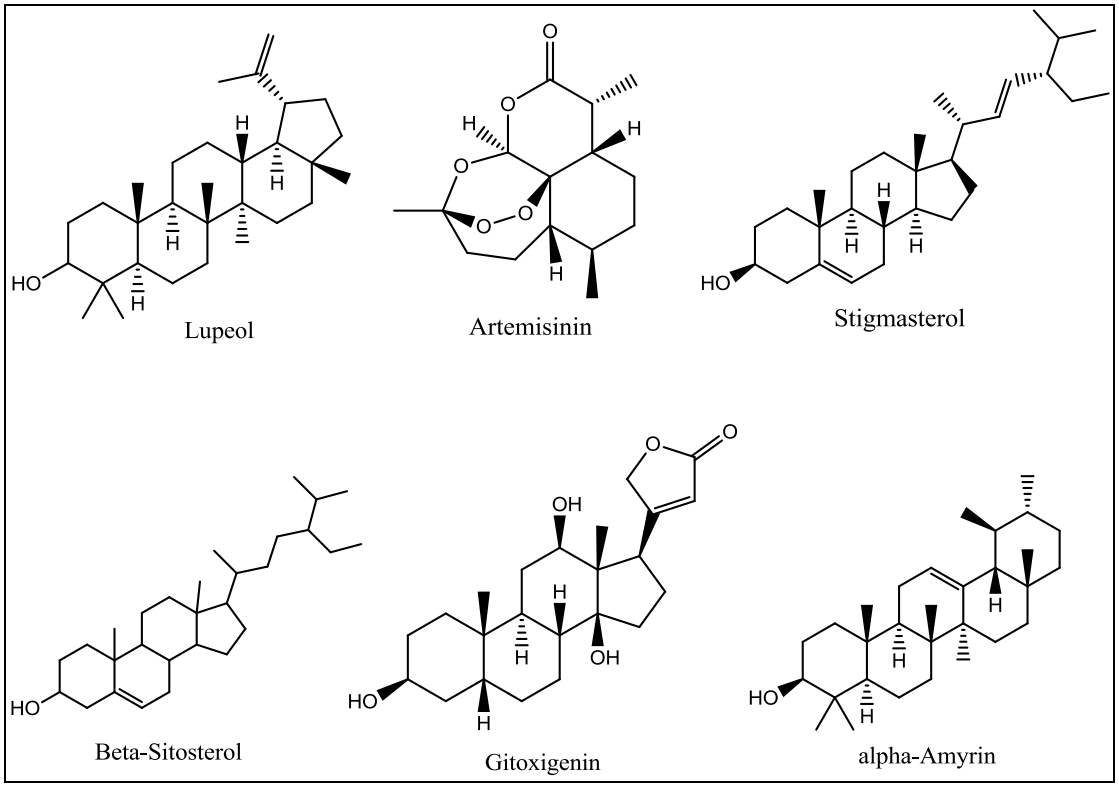


Fig. 28: Six phytochemicals namely (Sitosterol, Stigmasterol, Lupeol, Gitoxigenin, alpha-Amyrin and Artemisinin) out of the 70 reported natural components are validated as being active against cancerous cells.

Compounds such as sterols, triterpenes, esters and carboxylic acids were seen in the *Gundelia tournefortii* extracts in significant quantities. For example, about 10% of the typical sterols, sitosterol and stigmasterol (Fig. 28) were seen in the underivatized hexane extract (Table 12). It is reported that sitosterol act as inhibitor of tumor promotion *in vivo* and inhibit carcinogenesis [84,85]. Furthermore, stigmasterol significantly inhibit tumor promotion in two-stage carcinogenesis in mice [86]. A mixture of sitosterol and stigmasterol was shown to possess anti-inflammatory activity after topical application [87]. Therefore, it is expected that the presence of such sterols in *Gundelia tournefortii* would be of paramount importance to combat cancer.

Lupeol (Table 10 and Fig. 19), another phytochemical, which has never been reported to be present in *Gundelia tournefortii*, weighs about 10% in the hexane extract. It has been shown to act as a novel androgen receptor, which inhibits the proliferation of human prostate cancer cells by targeting β-catenin signaling Carcinogenesis [88].

In addition to the aforementioned four anticancer phytochemicals, Gitoxigenin (Fig. 26 and Table 11) which weighs about 1.5% in the of *Gundelia tournefortii* methanolic extract, was reported to exert significant anticancer activity against renal adenocarcinoma and other cancer cell lines with IC_{50} values in µM range [89]. Furthermore, α-Amyrin (Table 10 and Fig. 19) which exist in hexane extract in about 5.7%, was reported recently to have significant anticancer activity on four cancer cell lines (MCF-7, BEL-7402, SPC-A-1 and SGC-7901 cancer cell lines) with IC_{50} values 7.2±0.12, 8.2±0.29, 7.6±0.06 and 5.0±0.12 respectively [90].

The underivatized methanol extract revealed six carboxylic acids representing together about half of the whole extract percentage (48.9%). Artemisinin, an active promising photochemical was also noticed for the first time in *Gundelia tournefortii* extract comprising 2.39%. This compound is currently undergoing extensive investigation for the treatment of cancer [91]. Moreover, artemisinin was mentioned in over than 300 scientific papers as a potential compound in combating cancer.

4.2.2. *Gundelia tournefortii* methanol extract fractionation by preparative HPLC-PDA

An inch preparative reversed phase C18 column was used to collect as many as fractions to test their *in-vitro* assays. Fig. 29 shows the preparative chromatogram along with the UV-Vis of the most active fraction.

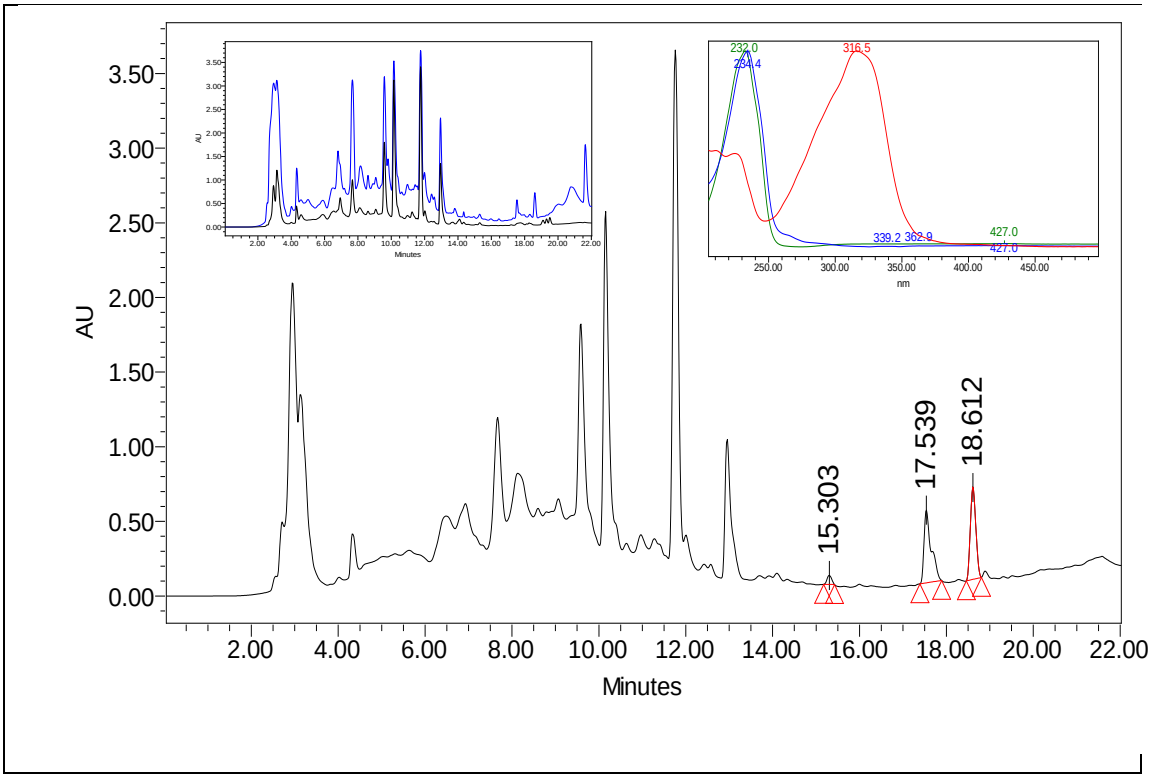


Fig. 29: Typical preparative HPLC chromatogram of *Gundelia tournefortii* methanol extract at wavelength 232 nm. An overlaid chromatograms at 210 nm (blue) and 232 nm (black) are shown at the left side of Fig. 27. Fractions retention times: I (2.1-7.4 minutes), II (7.4-8 minutes), III (8-9.2 minutes), IV (9.2-9.5 minutes), V (9.8-10.4 minutes), VI (11.6-11.8 minutes), VII (12.6-13.1 minutes) and VIII (13.2-21.1 minutes). The UV-Vis spectrum of active fraction VIII is located at the right side of Fig. 27.

After screening the *in vitro* anticancer activities of the eight preparative fractions, the cytotoxicity of fraction VIII was shown to be the highest against HCT-116 cancer cell line. The active fraction (VIII) contains three compounds (Fig. 29). The UV-Vis spectra of the 17.540 and 18.607 min eluted compounds possess maximum wavelengths of 234.4 and 232.0 nm respectively. The third compound which eluted at 18.6 minutes showed a maximum wavelength of 316.5 nm (Fig. 29).

4.2.3. Anticancer activity of the *Gundelia tournefortii* methanolic and hexane extracts

The effects of *Gundelia tournefortii* extracts on human colon cancer cell line (HCT-116) was determined using the MTT assay. HCT-116 cell line was exposed to *Gundelia tournefortii* methanolic, hexane and aqueous extracts (0-1000 µg/mL) for 22 hours. The EC_{50} values obtained by the MTT assay was 303.3 ± 12 µg/mL for the methanolic extract and 313.3 ± 18.6 µg/mL for the Hexane extract. However, aqueous extract reduced cell viability just to 74.8% at concentration of 1000 µg/mL. Fig. 30-32 summarize the results of MTT assay for *Gundelia tournefortii* methanol, hexane and aqueous extracts respectively.

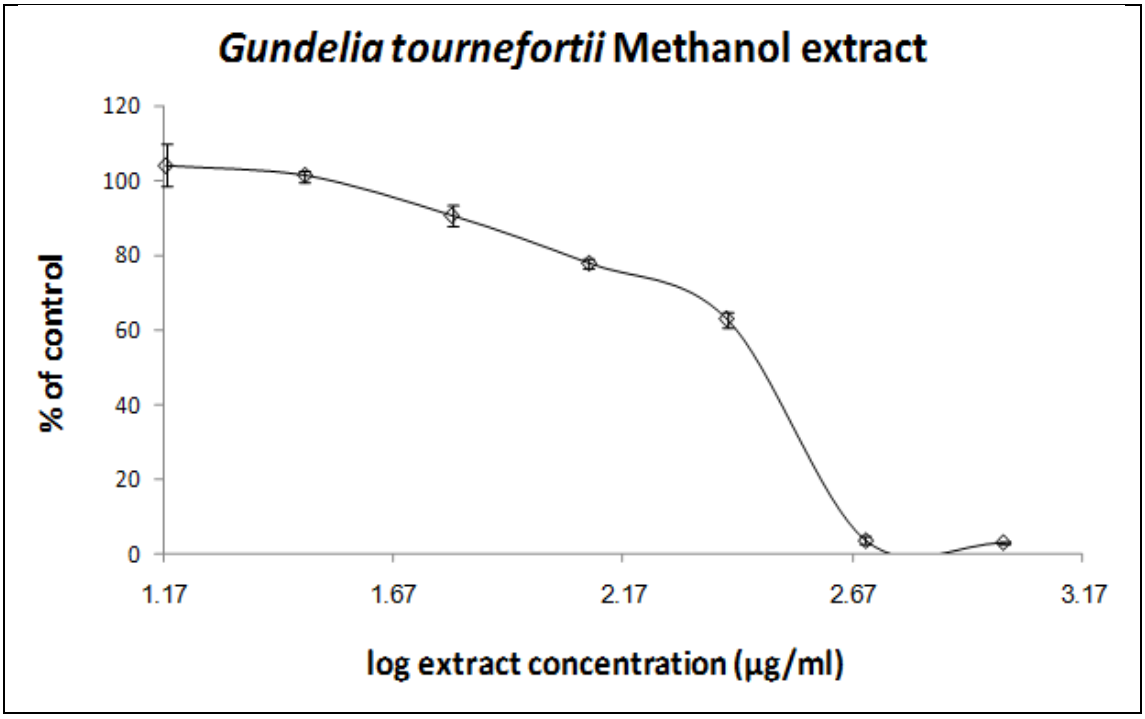


Fig. 30: Cytotoxic effect of *Gundelia tournefortii* methanol extract tested on HCT-116 cell line. HCT-116 cells were seeded in 96-well plate (20,000 cells/well), exposed to *Gundelia tournefortii* methanol extract for 22 hr. Cytotoxicity was determined by MTT assay. Each point represents the mean of the data from three independent experiments; bars represent the standard mean error (SEM) relative to the control.

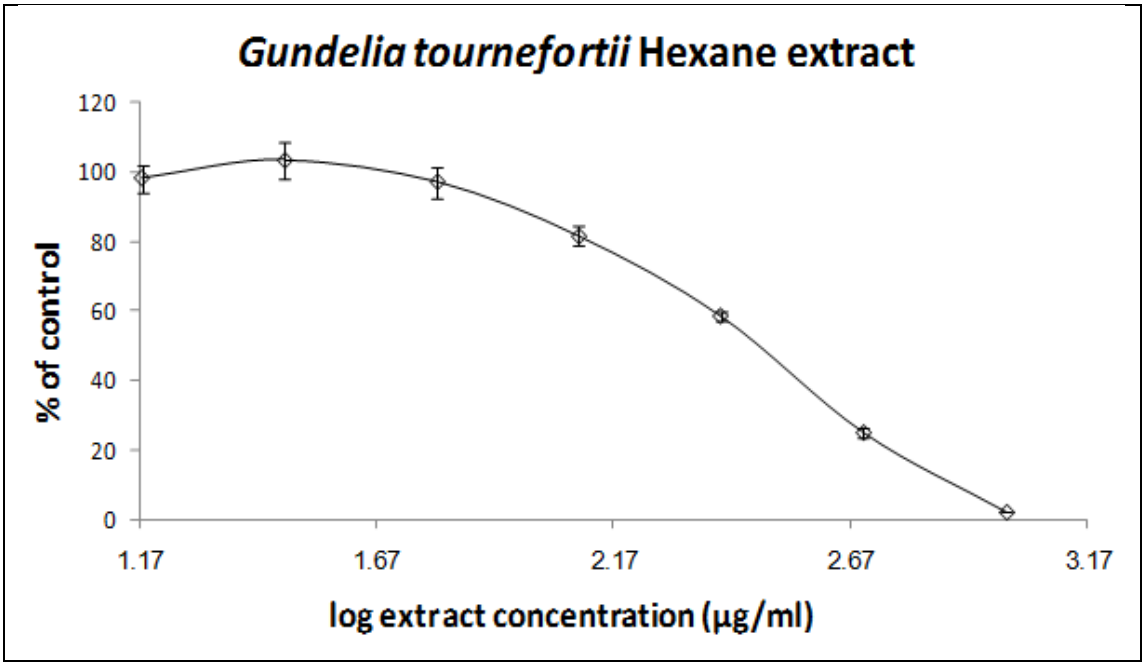


Fig. 31: Cytotoxic effect of *Gundelia tournefortii* hexane extract tested on HCT-116 cell line. HCT-116 cells were seeded in 96-well plate (20,000 cells/well), exposed to *Gundelia tournefortii* hexane extract for 22 hr. Cytotoxicity was determined by MTT assay. Each point represents the mean of the data from three independent experiments; bars represent the standard mean error (SEM) relative to the control.

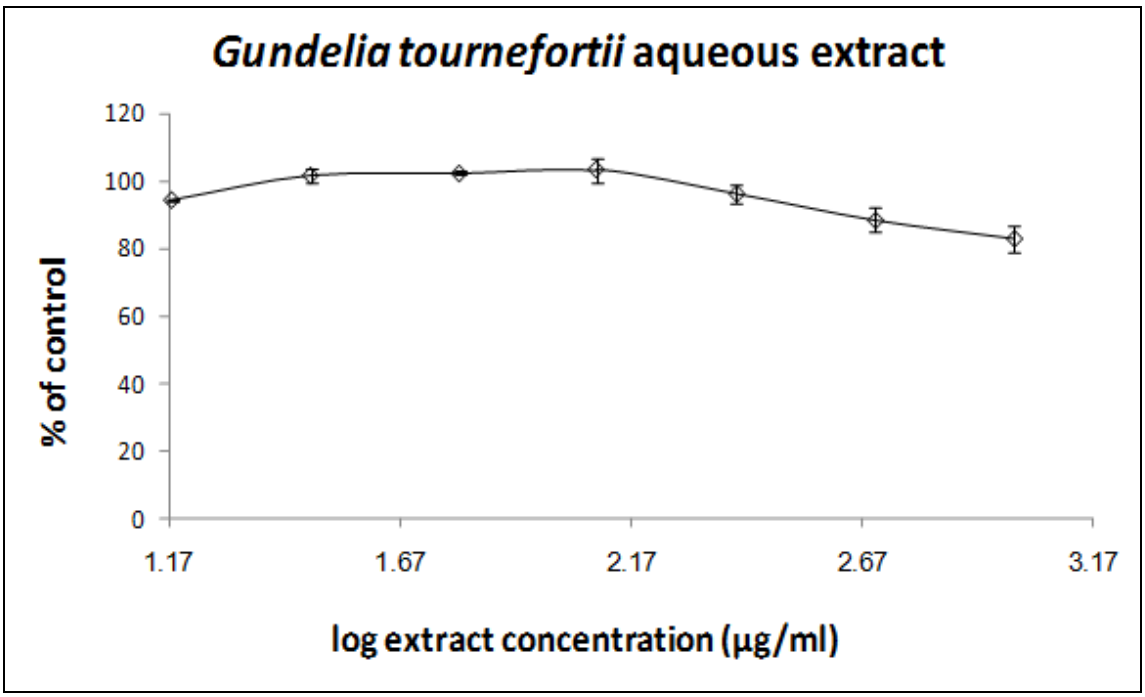


Fig. 32: Cytotoxic effect of *Gundelia tournefortii* aqueous extract tested on HCT-116 cell line. HCT-116 cells were seeded in 96-well plate (20,000 cells/well), exposed to *Gundelia tournefortii* aqueous extract for 22 hr. Cytotoxicity was determined by MTT

assay. Each point represents the mean of the data from three independent experiments; bars represent the standard mean error (SEM) relative to the control.

Prior to determine cytotoxicity for HCT-116 cell line (human colon cancer), fractions (from fraction I to fraction VIII) were evaporated and crude methanolic extract (0.1-1.3 mg/ml) were exposed for 22 hours to determine the EC_{50} by the MTT assay. Fraction VII is most active as seen from table 14. In the future, further preparative chromatography is required to separate about 50 mg of each compounds to run biological assays and disclose the identity of the anticancer responsible lead compound using ^{1}H-NMR, 2D-NMR and HR-MS.

Table 14: The activity of each fraction against HCT-116 cell line.

Fraction No	Viability (% of control) at 0.3 mg/ml
I	88.36 ± 3.26
II	87.48 ± 0.35
III	88.27 ± 4.82
IV	93.4 ± 2.01
V	81.5 ± 3.31
VI	85.74 ± 3.72
VII	47.45 ± 2.12
VIII	4.86 ± 0.31
Methanolic crude extract	35.23 ± 1.8

4.3.*Pimpinella anisum* plant

4.3.1. Preparative HPLC separation of *Pimpinella anisum* extract

The crude methanolic anise powder was highly soluble upon adding acetic acid to water (1%) which may point out for the presence of acidic functionalities in the crude mixture. Typical preparative HPLC chromatogram of crude anise and its corresponding overlaid UV-Vis spectra between 210-500 nm is shown in Fig. 31. The UV-Vis spectra showed peaks having multi absorption maxima's between the ranges of 219 to 342 nm. Six fractions were collected but fraction IV was the predominant one. However, the Fig. 33 revealed fraction V was the most pure fraction which upon reinjection using analytical HPLC-PDA gave pure peak having multi maxima's at

211.9, 246.2 and 298.5 nm respectively. This fraction proved to possess the highest anticancer activity on human prostate cancer cell line (PC-3).

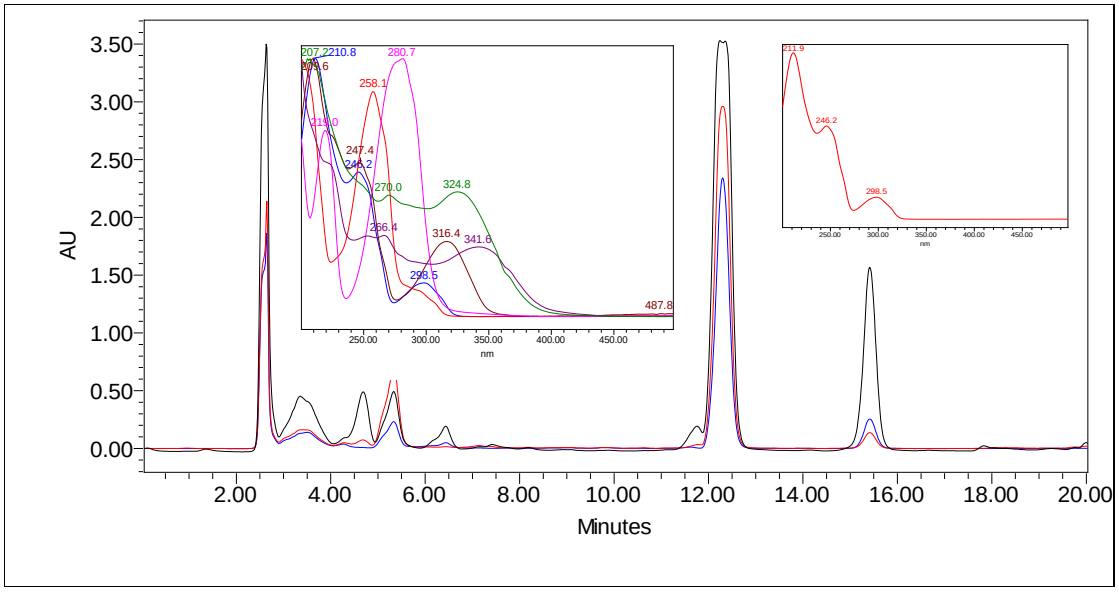
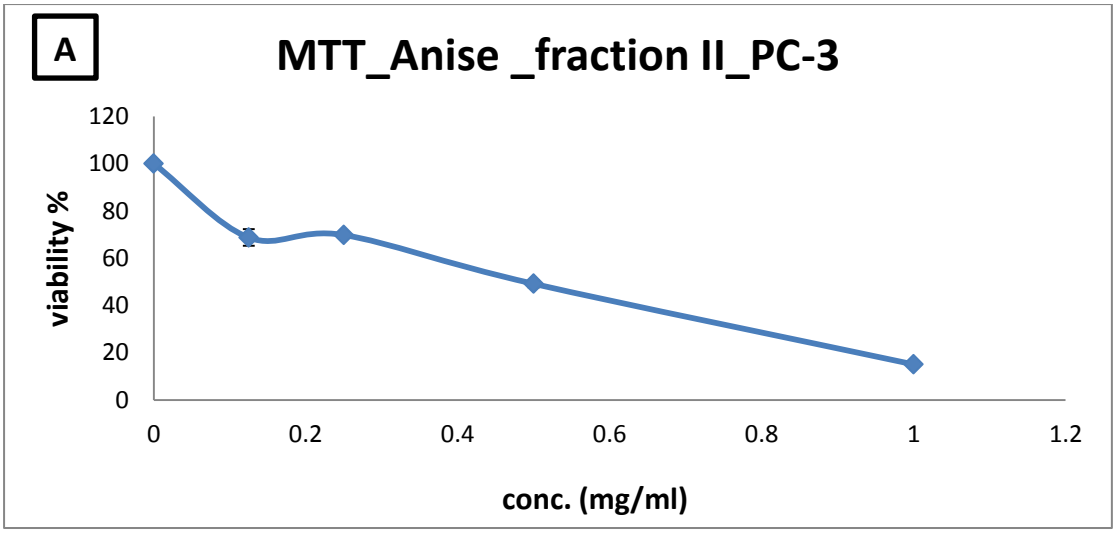


Fig. 33: Typical prep-HPLC chromatogram of crude anise. 1000 µl were injected at flow rate 20 ml/minutes. The monitoring wavelengths were at 220 (black), 280 (red) and 300 nm (blue). Collected fractions: I (1-4 minutes), II (4-6 minutes), III (6-12 minutes), IV (12-14 minutes), V (15-16 minutes) and VI (16-20 minutes). Overlaid UV-Vis spectra of all the eluted peaks are from 210-500 nm is to the left. The active peak UV-Vis of the pure fraction V is in right of the chromatogram.

4.3.2. Anticancer activity of the *Pimpinella anisum* methanolic extract

The extraction of *Pimpinella anisum* seeds by methanol, cytotoxicity experiments were conducted to reveal effectiveness of consuming anise in cancer prevention and treatment. The anise seeds methanolic extract (0-1 mg/ml), used to *in vitro* test to human prostate cancer cell line (PC-3), cytotoxicity was determined using MTT and LDH leakage assays as shown in Fig. 34-37.



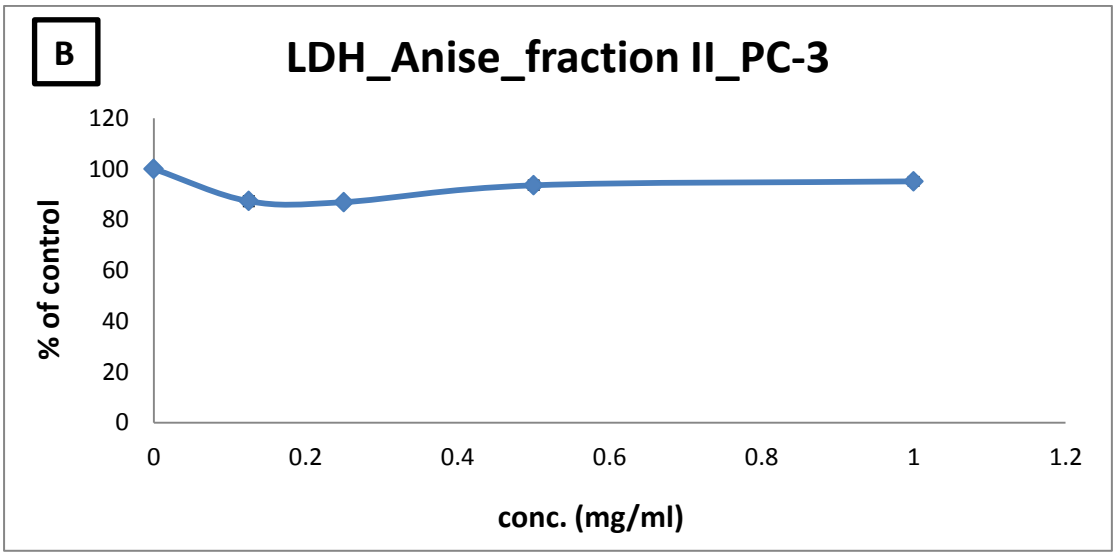
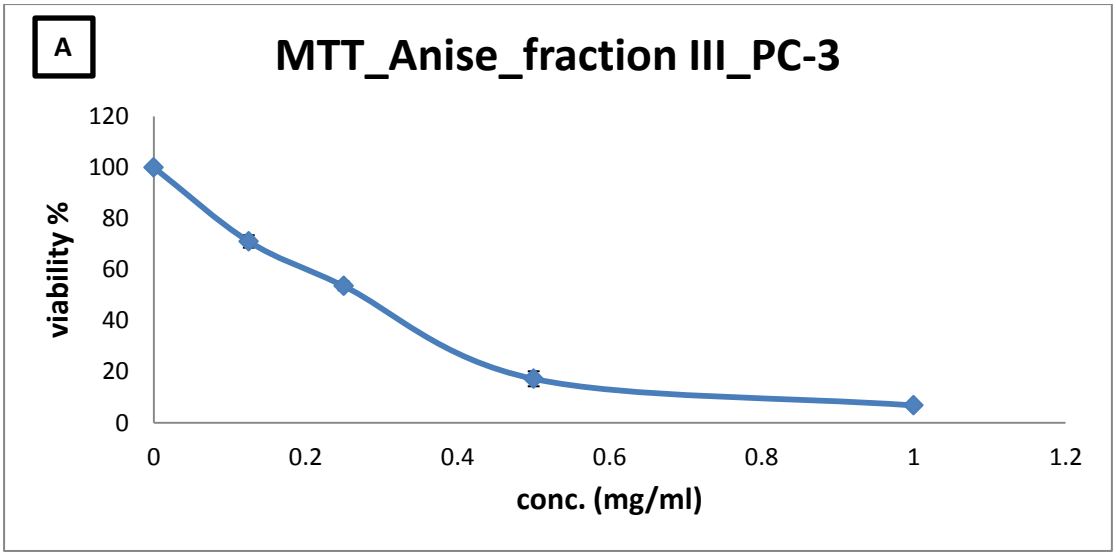


Fig. 34: MTT (A), LDH leakage (B) assays in PC-3 (Prostate carcinoma, human) after exposure to *anise* methanolic extract fraction II for 24 h. Data in A and B presented as percentage of control (*n=3*, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.



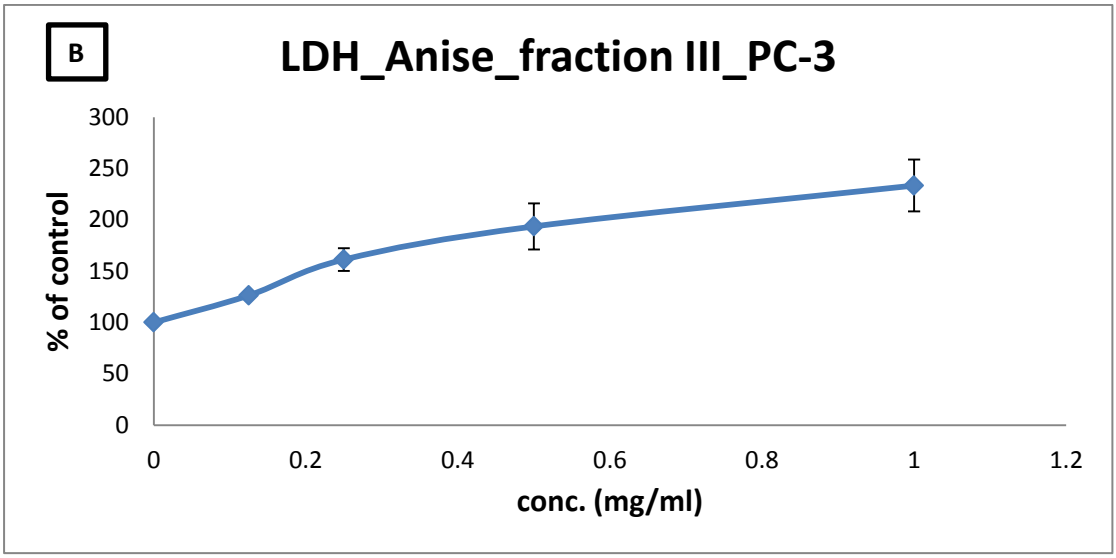
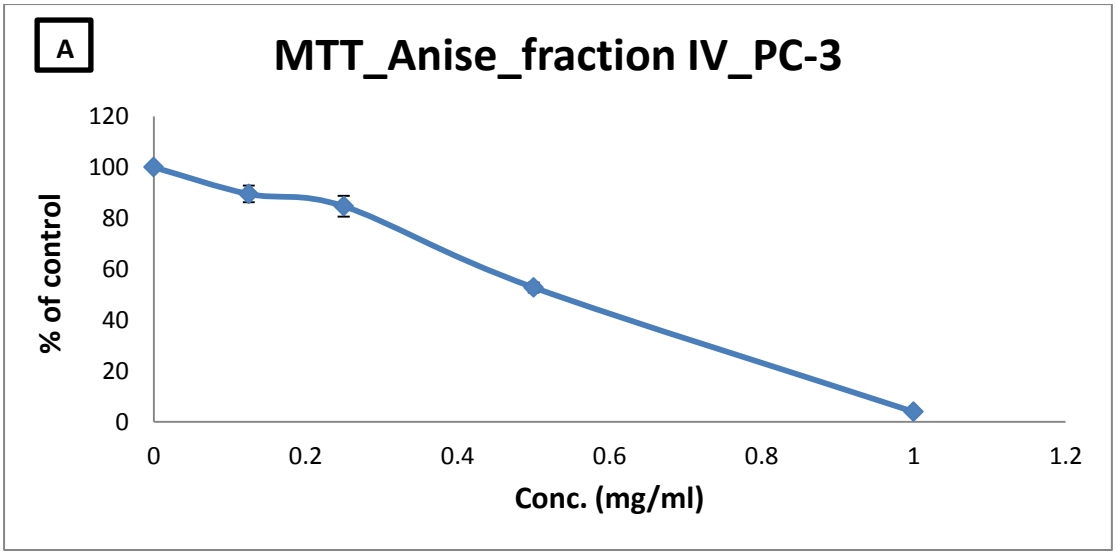


Fig. 35: MTT (A), LDH leakage (B) assays in PC-3 (Prostate carcinoma, human) after exposure to *anise* methanolic extract fraction III for 24 h. Data in A and B presented as percentage of control (*n=3*, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.



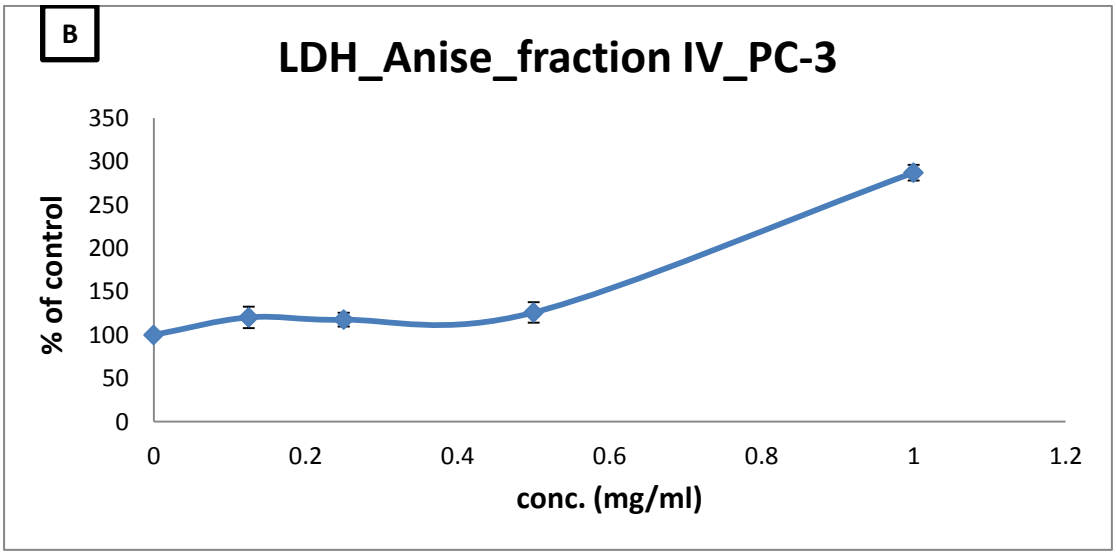


Fig. 36: MTT (A), LDH leakage (B) assays in PC-3 (Prostate carcinoma, human) after exposure to *anise* methanolic extract fraction IV for 24 h. Data in A and B presented as percentage of control (*n=3*, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

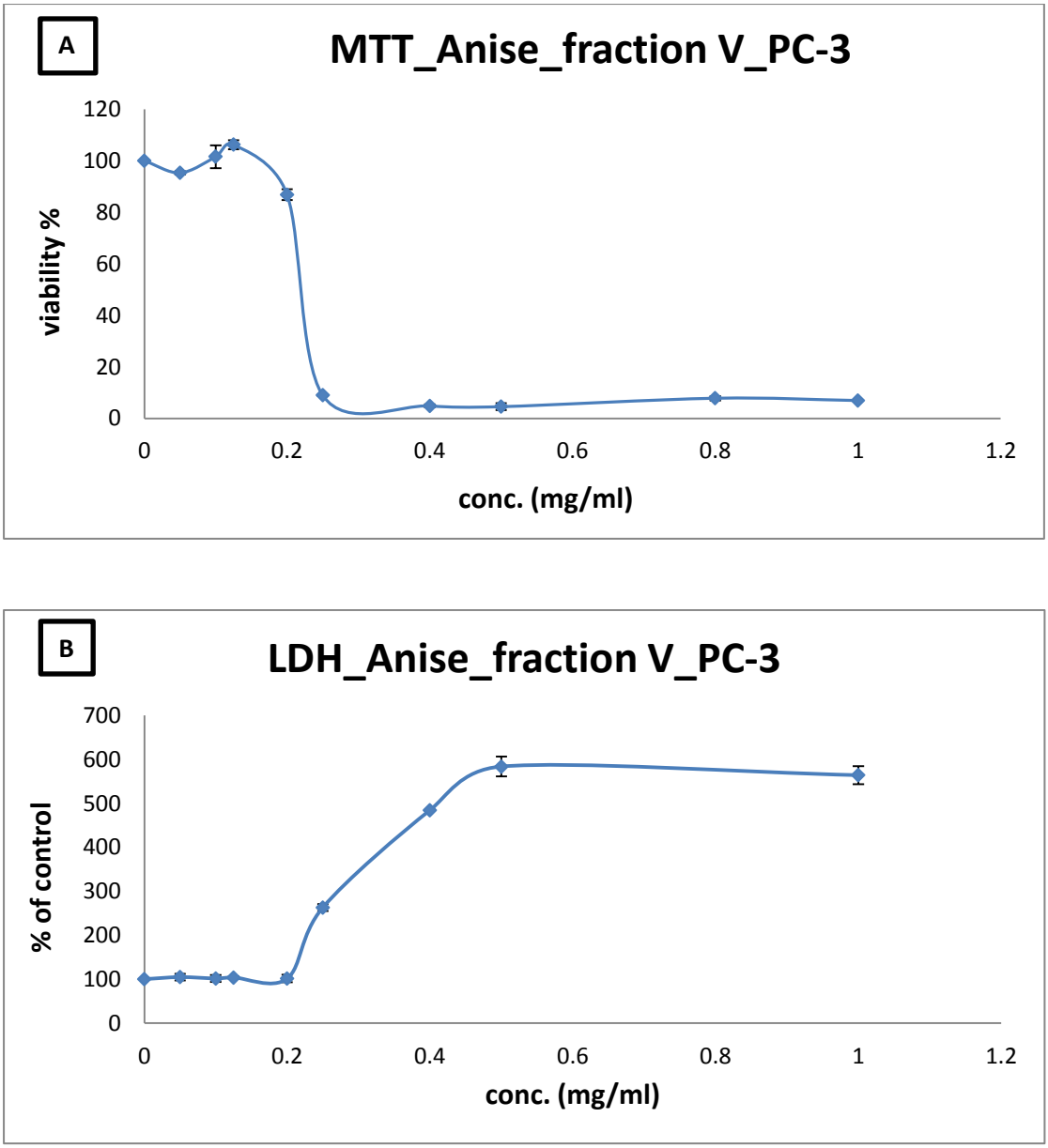


Fig. 37: MTT (A), LDH leakage (B) assays in PC-3 (Prostate carcinoma, human) after exposure to *anise* methanolic extract fraction V for 24 h. Data in A and B presented as percentage of control (*n=3*, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

The EC_{50} values obtained by the MTT assay was 0.5 ± 0.10 mg/ml for fraction II, and 0.3 ± 0.11 mg/ml for fraction III, and 0.49 ± 0.12 mg/ml for fraction IV, and 0.23 ± 0.10 mg/ml for fraction V, while the fraction I and VI is not active. Through the biological assay, the fraction V is more active than the other fraction.

^{1}H-NMR and HR-ESi-MS using the positive and negative modes was ran to the active compound from fraction V as seen in the appendix. Preliminary structural elucidation

of this compound indicate the presence of high molecular flavonoid compound. However, larger amount of this compound followed by silylation and GC-MS analysis along with ^{13}C-NMR, COSY and HMQC 2D-NMR are needed to be executed to fully elucidate the exact structure.

4.4.*Ephedra alata* plant

4.4.1. Preparative HPLC fractionation of *Ephedra alata* extract

The analytical HPLC-PDA chromatographic profile of *Ephedra alata* methanolic extract at 350 nm is shown in Fig. 38-A. Almost all the main peaks are sharing maximum wavelength of 348.5 nm-352.1 nm. These types of compounds are very close to isomeric flavonoid glycosides such as symplocoside, kaempferol 3-O-rhamnoside 7-O-glucoside, isovitexin 2-O-rhamnoside, which may contribute significantly to the antioxidant activity and therefore the anticancer of the plant [92].

The preparative chromatogram of 1000 µl injection of *Ephedra. alata* methanolic extract into inch reversed phase column is shown in Fig. 38-B.

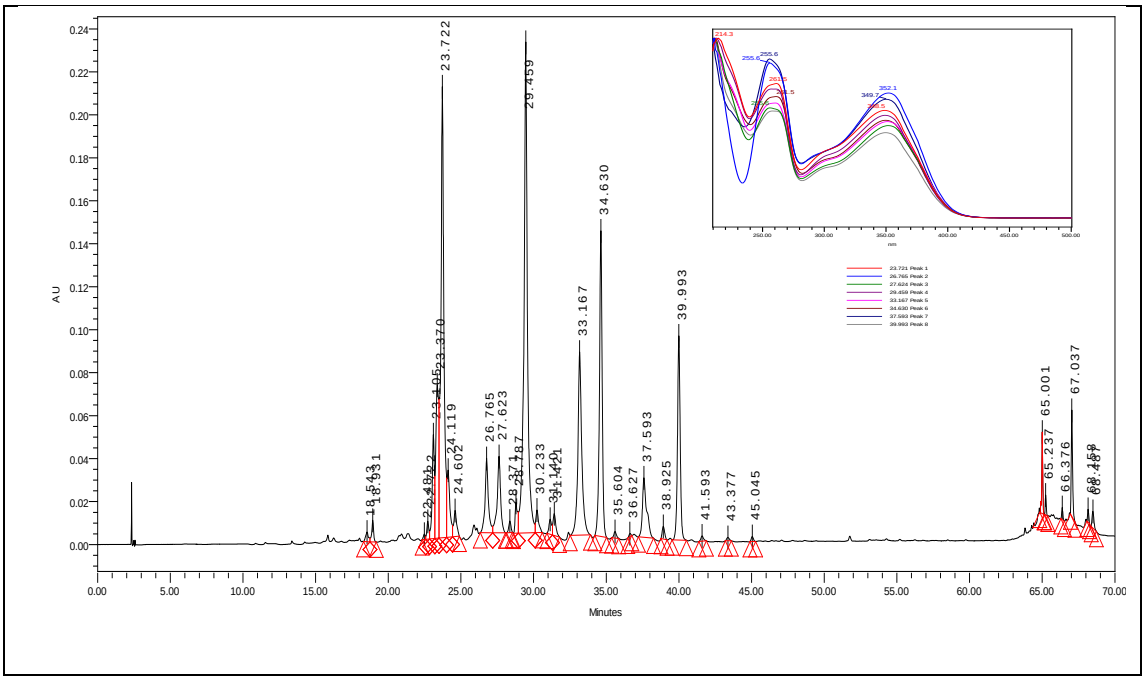


Fig. 36-A: Chromatograms of flavonoids mixture from methanolic extract of *Ephera alata* at 350 nm. The overlaid UV-Vis spectra of the main peaks are depicted at the right corner of chromatogram.

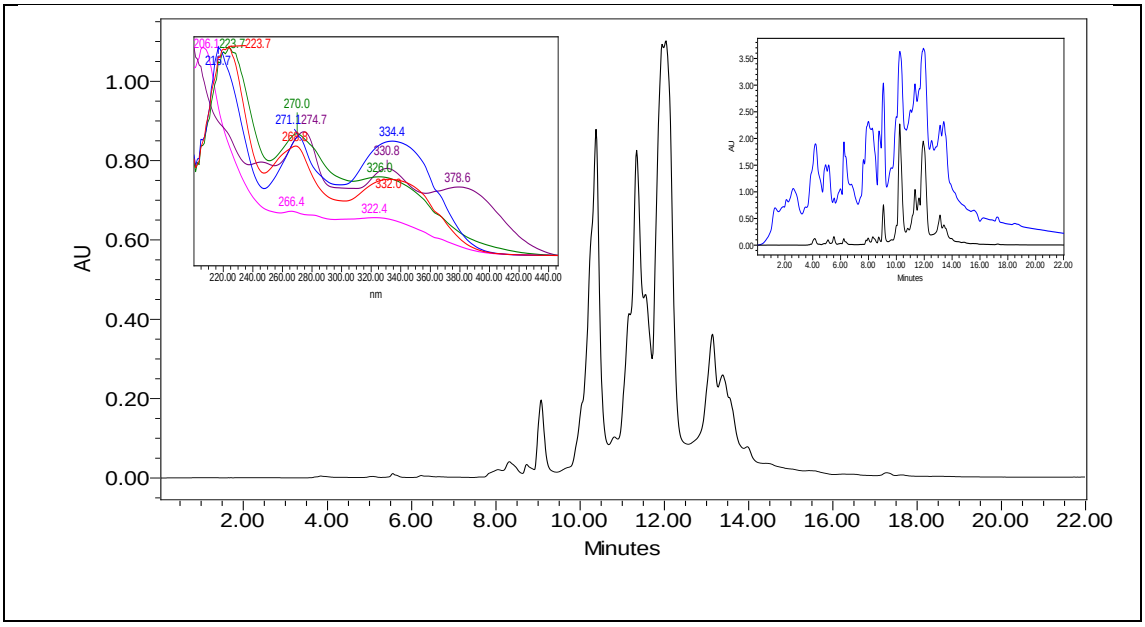


Fig. 38-B: Preparative chromatogram of *Ephedra alata* methanol. The overlaid chromatogram at 270 nm (black) and 210 nm (blue) are shown at the right corner of the fig. 38-B. The UV-Vis spectra are located at the left corner of the figure.

There are many broad peaks appeared in the chromatogram above. Several fractions were collected and their cytotoxicity effect is currently under investigation.

4.4.2. Anticancer activity of *Ephedra alata* methanolic extract

Ephedra alata plant methanol extract is prepared to disclose the effectiveness of inhibit growth of tumors. The human liver cancer cell-line (HepG2) was exposed to *Ephedra alata* crude methanol extract (0-800 µg/mL) for 24 h, and cytotoxicity was determined using the MTT assays. The EC_{50} values obtained by the MTT assay was 25 ± 1.2 µg/ml.as shown in Fig. 39.

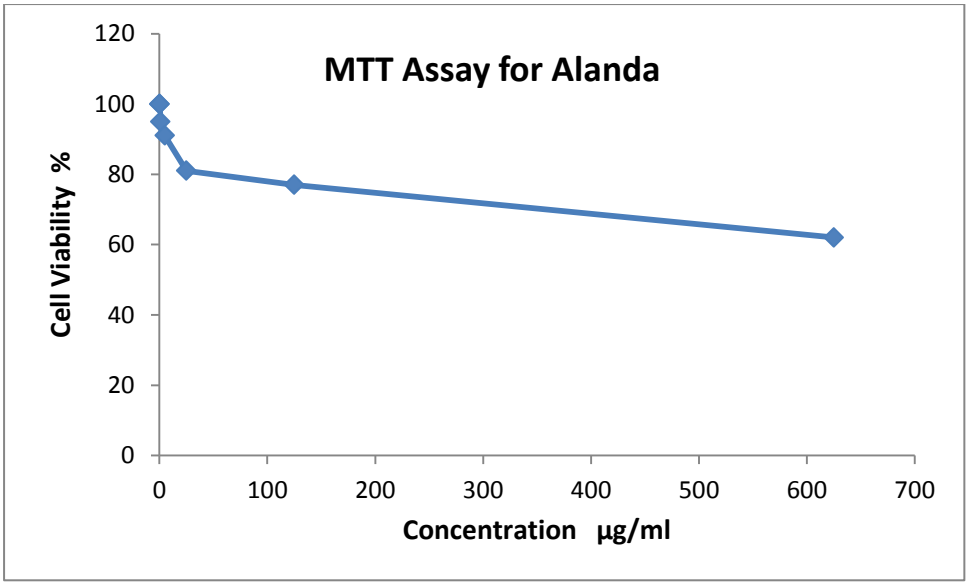


Fig. 39: MTT assays in HepG2 (human liver cancer cell-line) after exposure to *Ephedra* methanolic crude extract for 24 h. Data in A presented as percentage of cell viability (*n=3*, each value represents the mean of triplicate) ±SEM; SEM: standard error mean.

Chapter Five

Conclusion and Recommendation

5.1. Conclusion

Preparative HPLC-PDA chromatographic fractionation of selected four Palestinian plants were successfully achieved and were investigated for their *in-vitro* anticancer activities. To our knowledge, this study is the first to highlight the anticancer activity of *Cuscuta palaestina* and *Gundelia tournefortii* fractions and pure compounds. Both methanolic and hexane extracts have shown considerable anticancer capacity against HCT-116 cancer cell. Methanolic extract of *Cuscuta palaestina* for example exerted a significant antiproliferative effect while the aqueous extract of *Gundelia tournefortii* was inactive. GC-MS revealed miscellaneous phytochemicals in methanolic and hexane extracts for both plant. Sesamin phytochemical could be the major source for the anticancer activity of *Cuscuta palaestina* extract. The GC-MS and PLC-PDA findings advocate that Sesamin content in the methanolic extract of *Cuscuta palaestina* is three times more than that from sesame seed. Two others Phytosterols namely Campesterol and Stigmasterol that are well known for their anticancer activity were detected in the methanolic extract of *Cuscuta palaestina* but not in hexane extract, while the *Gundelia tournefortii* contained Stigmasterol in the nonderivatized hexane extract and silylated BSTFA methanol and hexane extract alike. *Gundelia tournefortii* showed another potent chemicals such as β-Sitosterol, Lupeol, Gitoxigenin, α-Amyrin and Artemisinin which may act synergistically and therefore would be of paramount importance to prevent and to combat cancer. The results of the current study are coherent with the acquired folk traditional reputation that is translated through the survey conducted on participants who are eating Akoub on regular basis and believe it to possess anticancer benefits.

The methanolic extract of anise seeds has also shown a significant anticancer effect on human prostate cancer (PC-3 cell line). Thus, anise could be one of the foods and drinks (herbal tea) that attribute to cancer prevention and treatment. Preparative HPLC of fraction V and its *in vitro* test showed promising anticancer activity. Moreover, *Ephedra alata* crude methanolic extract has shown antiproliferative activity against human liver carcinoma cell line (HepG2). According to the HPLC-PDA chromatogram and spectra of the major separated peaks of *Ephdra alata* crude methanolic extract, we believe that isomeric flavonoid glycosides may contribute significantly to the anticancer activity of this plant.

5.2. Recommendation

The following are some suggestions for future investigations.

1. Scaling-up sesamin secondary metabolite from *Cuscuta palaestina* by using preparative chromatography to run *in-vitro* and *in-vivo* anticancer assays on different cell lines and animal models. Moreover, the percentages of sesamin from different resources can be explored in comparison to sesame seeds.
2. Elucidation of the exact structure of the active compound in *Pimpinella anisum, Ephedra alata* and in fraction VIII for *Gundelia tournefortii* by using spectroscopic techniques such as high resolution-mass spectroscopy (LC-QTOF) and 2D-NMR.
3. Systematically investigate the synergistic effects of combining different fractions and/or diffrent pure compounds from each plant and examining their *in-vitro* and *in-vivo* assays.
4. The percentage of the active compounds could be increased by designing biotech engineered approaches.

6. References

1. Bingham, S., Riboli, E., (2004). Diet and cancer the European prospective investigation into cancer and nutrition. *Nature Rev Cancer* 4, 206- 215.
2. Stewart, B.W. and Kleihues, P. (2014). International Agency for Research on Cancer. *World Cancer Lyon Report*, 351.
3. Bharat, B.A., Shishir, S., (2006). Molecular targets of dietary agents for prevention and therapy of cancer. *BiochemPharmacol* 71, 1397-1421.
4. Anand P., Kunnumakkara A.B., Sundaram C., Harikumar K.B., Tharakan S.T., Lai OS, Sung B., and Aggarwal B.B., (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharm Res,* 25:2097-2116.
5. Bailar J.C., Gornik H.L., (1997). Cancer undefeated. *N Engl J Med*, 336:1569-1574.
6. Husemann Y., Geigl J.B., Schubert F., Musiani P., Meyer M., Burghart E., Forni G., Eils R., Fehm T., Riethmuller G., Klein C.A., (2008). Systemic spread is an early step in breast cancer. *ICBN*. Vol. 13:58-68.
7. (http://globocan.iarc.fr/Pages/fact_sheets_population.aspx)
8. Rebecca L. Siegel M., (2015). Cancer Statistics.*CA CANCER J CLIN*. Vol 65:5–29.
9. Schoental, R., (1965). Toxicology of natural products. Food Cosmetics Toxicol., *ICBN*. VOL. 3(4), 609–620.
10. Rodney C., Toni M., Norman G. (2000). Natural products (secondary metabolites). Biochemistry and Molecular biology of plants, B. Buchanan, W. Gruissem, R. Jones, Eds. American Society of plant physiologists.
11. Karla R., Diego H., (2016). Elicitation, an Effective Strategy for the Biotechnological Production of Bioactive High-Added Value Compounds in Plant Cell Factories. *Review. Molecules* 21,182.
12. Hyun J., Eiichiro O., Honoo S., (2009) .Metabolic Engineering of Lignan Biosynthesis in Forsythia Cell Culture. Plant Cell Physiol. 50(12): 2200-2209. Doi:10.1093/pcp/pcp156.
13. Eisenberg. D.M., Davis R.B., Ettner S.L., Appel S., Wilkey S., Van Rompay M, Kessler RC., (1998). Trends in alternative medicine use in the United States 1990 – 1997: results of a follow up national survey. *Journal of American Medical association* 280, 1569 – 1575.

14. Santosh Kumar Singh ., (2015) Traditionally used Medicinal Plants as Alternative Source for Future Anticancer Drugs. *ISSN* 2. P. 321 – 919.
15. Zaid H., Rayan A., Said O., Saad B., (2010). Cancer Treatment by Greco-Arab and Islamic herbal medicine. *The Open Nutraceuticals Journal*., 3,P 203-212.
16. Zaid H., Rayan A., (2010). Cancer Treatment by herbal medicine. *The Open Nutraceuticals Journal*, 3, 215-222.
17. Cragg GM., Newman DJ., (2005). Plants as a source of anticancer agents. *J. Ethopharmacol* 100, 72–79.
18. Tan G., Gyllenhaal C., Sorjarto D.D., (2006). Biodiversity as a source of anticancer drugs. *Curr. Drug Targets* 7, 265–277.
19. Farnsworth NR., Akerele O., Bingel AS., Soejarta DD., Eno Z., (1985). Medicinal plants in therapy. *Bull World Health Organ* 63, 965-981.
20. Madhuri S., Pandey G., (2009). Some anticancer from medicinal plants of foreign origin. *Curr Sci* 96, 779-783.
21. Meyer B.N., Ferrigni N.R., Putnam J.E., Jacobsen L.B., Nichols D.E., McLaughlin J.L., (1982). A convenient general bioassay for active plant constituents. *Planta Med.* 45, 31-34.
22. Verpoorte R., (2000). Pharmacognosy in the new millenium: lead finding and biotechnology. *J Pharm Pharmacol* 52, 253–262.
23. Jawad Alzeer, Balayeshwanth R., Rami Arafeh, (2014), The influence of extraction solvents on the anticancer activities of Palestinian medicinal plants. *Journal of Medicinal Plant Research*. 8(8), pp. 408-415.
24. Musallam, Duwayri M., and Shibli R. A., (2011). "Micropropagation of *caper* (*Capparis spinosa* L.) from wild plants", Functional Plant Science and Biotechnology, vol. 5 *(Special Issue 1), pp*. 17-21.
25. Bassam Abu-Shanab, Ghaleb Adwan, (2005) Antibacterial Activity of *Rhus coriaria* L. extracts growing in Palestine. *Journal of The Islamic University of Gaza, (Natural Sciences Series),*Vol.13, No.2, P147-153.
26. Adriana B., (2001). Natural products in anticancer therapy. *PubMed* (01)00063.
27. Shah U., (2013). Novel anticancer agents from plant sources. *Chinese Journal of Natural Medicines*, 11(1): 0016−0023.

28. Rajandeep Kaur. Plants as a source of anticancer agents. *J. Nat. Prod. Plant Resour*., 2011, 1 (1): 119-124.
29. Malleshappa N., Noolvi M., (2011) Natural sources as potential anti-cancer agents: *A review, ISSN*. 0975-0185.
30. Gower D.B., Makin H.L., (2009). Steroid analysis (2nd ed.). *Dordrecht: Springer. ISBN*. 9781402097744.
31. SticherO., (2008). Natural product isolation. *Nat. Prod. Rep.*, p 25, 517–554.
32. Ghisalberti E., (2007). Detection and Isolation of Bioactive Natural Products, *pp*. 11–76.
33. Franz Bucar, (2013). Natural product isolation – how to get from biological material to pure compounds. *Review, Nat. Prod. Rep*., 30, 525-545.
34. Mateus E., Barata R. C., Zrostlikova J., (2010). Isolation of natural product by Chromatography technique, 1217, 1845–55.
35. Mihai C., (2015). A Phylogenetically Based Infrageneric Classification of the Parasitic Plant Genus Cuscuta (Dodders, Convolvulaceae), Systematic Botany. *Book*, 40(1): pp. 269–285.
36. Hassler M. (2016). World plants Synonymic checklists of the vascular plant of the world. *ISSN*. 2405-8858.
37. Mishra JS., (2009). Biology and Management of *Cuscuta* species. *Indian J. Weed Sci.* 41 (1,2) : 1-11.
38. Rebecca S., Ahmadin J., (2014-2016). Colorectal cancer facts and figures publication of the American Cancer Society, Atlanta, Georgia.
39. Majid S., Asadi S., Mahmoud RK., (2013). Gundelia: A Systematic Review of Medicinal and Molecular Perspective. *PJBS*, 16(21): 1238-1247.
40. Wijesekera, R. O., (1991). Is there an industrial future for phytopharmaceutical drugs An outline of UNIDO programmes in the sector. *J Ethnopharmacol*, 32(1-3), 217-224.
41. Ali-Shtayeh, M. S., Jamous, R. M., Al-Shafie, J. H., Elgharabah, W. A., Kherfan, F. A., Qarariah, K. H., Khdair, I. S., Soos, I. M., Musleh, A. A., Isa, B. A., Herzallah, H. M., Khlaif, R. B., Aiash, S. M., Swaiti, G. M., Abuzahra, M. A., Haj-Ali, M. M., Saifi, N. A., Azem, H. K., & Nasrallah, H. A. (2008). Traditional knowledge of wild edible plants used in Palestine (Northern West Bank): a comparative study. *J Ethnobiol Ethnomed*, 4, 13.

42. Salehi M.M, (2010). Medical plants and phytotherapy, Donyay Taghziah *Press, Tehran, Iran*, vol. 1234-242.
43. Rana A. Z., (1996). Medicinal plants, *Tehran University press, Iran*, vol 2, 212-220.
44. Sleman K., Mahmoud R. and Anwar R., (2013). Anticancer Activity of Anise *(Pimpinella anisum L.)* Seed Extract. *The Open Nutraceuticals Journal, 6, 1-5.*
45. Nidal J., and Fatima H., and Anas A., (2015). Preliminary Phytochemical Screening, Quantitative Estimation of Total Flavonoids, Total Phenols and Antioxidant Activity of *Ephedra alata* Decne. *ISSN :* 2028-2508.
46. Larry Walker, (2003). *Ephedra in perspective – a Current Review, Phytother. Res. 17, 703-712.*
47. Hong-Xia Li, Ming-Yu Ding, (2001) Separation and Determination of Epedrine Alkaloids and Tetramethylpyrazine in *Ephedra sinica* Stapf by GC-MS. *Journal of chromatographic Science, Vol. 39.*
48. Aden D.P., Fogel A., Plotkin S., Damjanov I., Knowles BB., (1979). Controlled synthesis of HbsAg in a differentiated human liver carcinoma-derived cell line. *Nature;* 282:615-7.
49. Snyder L. R., and Kirkland (1979). Introduction to modern liquid chromatography 2nd ed., New york: Wiley.
50. Grob R. L., and Barry E. F., (2004). Modern Practice of Gas Chromatography, 4th ed., New York: Wiley-Interscience.
51. Atkins and Jones, Bibliographical source: Pg. 702 of , Chemical Principles, 3rd Edition and Pg. 484 of Bruice, Organic Chemistry, 4th Edition.
52. Adams F., Gijbels R., and Van Grieken R., (1988). Inorganic Mass Spectrometry, John Wiley & Sons, Inc., New York.
53. Syed Z. H., (2014). GC-MS principle, Technique and its application in Food Science. *ISSN*, 2250-1770.
54. Kadan S., Rayan M., Rayan A., (2013). Anticancer Activity of Anise (Pimpinella anisum L.) Seed Extract. *The Open Nutraceuticals Journal* 6, 1-5.
55. Zaid H., Raiyn J., Nasser A., Saad B., Rayan A., (2010). Physicochemical properties of natural based products versus synthetic chemicals. *The Open Nutraceuticals Journal*. 3, 194-202.

56. Michael W., and Ludger W., (1992). Quinolizidine alkaloids in *Genista acanthoclada* and its holoparasite, *Cuscuta palaestina. Journal of Chemical Ecology,* Vol. 3,
57. Wink M., (1992). Allelochemial properties or the raison d'etre of alkaloids. In G. Cordell the alkaloids. In press. New York.
58. Coruh N., Sagdicoglu A.G., (2005). Gundelia tournefortii L. extracts and inhibition on glutathione-S-transferase activity. *Food Chemistry* 1249-1253.
59. Khanzadeh F., Rahmani. F., (2012). Physiochemical Properties of Gundelia tournefortii L. Seed Oil. *J. Agr. Sci. Tech*. Vol. 14:1535-1542.
60. Azeez O.H., and Kheder A. E., (2012). Effect of Gundelia tournefortii on some biochemical parameters in dexamethasone-induced hyperglycemic and hyperlipidemic mice. *Iraqi Journal of Veterinary Sciences*, Vol. 26, No. 2, (73-79).
61. Jovana D., Snezana O., (2015). Compositional characteristics of the essential oil of *Pimpinella anisum* which grows in Serbia. *Botanica Serbica* 39 (1), 09-14.
62. Aisha Z. I., (2015). In vitro antimicrobial activity of Anise seed *(Pimpinella anisum L.). International Journal of Advanced Research, V 3, Issue 1, 359-367.*
63. Abdalsalam K., Badlaa L., Hilal Z., and Bashar S., (2015). In vitro assessments of cytotoxic and cytostatic effects of *Asparagus aphyllus, Crataegus aronia,* and *Ephedra alata* in monocultures and co-cultures of Hepg2 and THP-1 derived macrophages. *Phcog*. V. 5. *Issue 3.*
64. Larry W., (2003). Ephedra in perspective – a Current Review, Phytother. *Res.* 17, 703-712.
65. Hong-Xia L., Ming-Yu D., (2001). Separation and Determination of Epedrine Alkaloids and Tetramethylpyrazine in *Ephedra sinica Stapf* by GC-MS. *Journal of chromatographic Science,* Vol. *39.*
66. Gurley J., Wang P., and Garder S., (1998). Ephedrine-Type alkaloid content of nutritional supplements containing *Ephedra sinica* (Ma-huang) as determined by high performance liquid chromatography. *Journal of Pharmaceutical sciences,* Vol. *87* No. *12.*

67. Patel S., Gheewala N., Suthar A., Shah A., (2009). *In vitro* Cytotoxicity activity of *Solanumnigrum* extracts against *Hela* and *Vero* cell lines. *Int J Pharm Pharm Sci* 1, 38-46.
68. Feleszko W., Mlynarczuk I., Olszewska D., Jalili A., Grzela T., Lasek W., (2002). Lovastatin potentiates antitumor activity of doxorubicin in murine melanoma via an apoptosis-dependent mechanism. *Int J Cancer*. 100, 111-118.
69. Kadan S., Saad B., Sasson Y., & Zaid H., (2013). In Vitro Evaluations of Cytotoxicity of Eight Antidiabetic Medicinal Plants and Their Effect on GLUT4 Translocation. *Evid Based Complement Alternat Med*, 549345.
70. Kadan S., Rayan M., & Rayan A., (2013). Anticancer Activity of Anise (Pimpinella anisum L.) Seed Extract. The Open Nutraceuticals Journal, 6, 1-5.
71. Stalling D. L., Gehrke C. W., and Zumwalt R. W., (1968). A new silylation reagent for amino acids bis(trimethylsilyl)trifluoroacetamide (BSTFA). *Biochem Biophys Res Commun*, 31(4), 616-622.
72. Li J.H., Awad A.B., Fink C.S., Wu Y.W., Trevisan M., Muti P., (2001). Measurement variability of plasma beta-sitosterol and campesterol, two new biomarkers for cancer prevention. *Eur J Cancer Prev.* 10 (3), 245-249.
73. Choi J. M., Lee E. O., Lee H. J., Kim K. H., Ahn K. S., Shim B. S., Kim N. I., Song, M. C., Baek, N. I., Kim S. H., (2007). Identification of campesterol from Chrysanthemum coronarium L. and its antiangiogenic activities. *Phytother Res* 21, 954-959.
74. Ali H., Dixit S., Ali D., Alqahtani S. M., and Alarifi S., (2015). Isolation and evaluation of anticancer efficacy of stigmasterol in a mouse model of DMBA-induced skin carcinoma. *Drug Des Devel Ther, 9*, 2793-2800.
75. Yasukawa K., Takido M., Matsumoto T., (1991). Sterol and triterpene derivatives from plants inhibit the effects of a tumor promoter, and sitosterol and betulinic acid inhibit tumor formation in mouse skin two-stage carcinogenesis. *Oncology* 48, 72-76.
76. Hirose N., Inoue T., Nishihara K., Sugano M., Akimoto K., Shimizu S., Yamada H., (1991). Inhibition of cholesterol absorption and synthesis in rats by sesamin. *J Lipid Res* 32, 629–638.

77. Kong X., Yang J.R., Guo L.Q., Xiong Y., Wu X.Q., Huang K., Zhou Y., (2009). Sesamin improves endothelial dysfunction in renovascular hypertensive rats fed with a high-fat, high-sucrose diet. *Eur J Pharmacol* 620, 84–89.
78. Harikumar K.B., Sung B., Tharakan S.T., Pandey M.K., Joy B., Guha S., Krishnan S., Aggarwal B.B., (2010). Sesamin manifests chemopreventive effects through the suppression of NF-kappa B-regulated cell survival, proliferation, invasion, and angiogenic gene products. *Mol Cancer Res* 8, 751–761.
79. Akl M.R., Ayoub N.M., Sylvester P.W., (2012). Mechanisms mediating the synergistic anticancer effects of combined gamma-tocotrienol and sesamin treatment. *Planta Med* 78, 1731–1739.
80. Truan J.S., Chen J.M., Thompson L.U., (2012). Comparative effects of sesame seed lignan and flaxseed lignan in reducing the growth of human breast tumors (MCF-7) at high levels of circulating estrogen in athymic mice. *Nutr Cancer*, 64, 65–71.
81. Tanabe H., Kuribayashi K., Tsuji N., Tanaka M., Kobayashi D., Watanabe N., (2011). Sesamin induces autophagy in colon cancer cells by reducing tyrosine phosphorylation of EphA1 and EphB2. *Int J Oncol*, 39, 33–40.
82. Wang H.M., Cheng K.C., (2010). Obtusilactone A and (−)-sesamin induce apoptosis in human lung cancer cells by inhibiting mitochondrial Lon protease and activating DNA damage checkpoints. *Cancer Sci*, 101, 2612–2620.
83. Asadi-Samani M., Rafieian-Kopaei M., and Azimi N., (2013). Gundelia: a systematic review of medicinal and molecular perspective. *Pak J Biol Sci*, 16(21), 1238-1247.
84. Raicht R. F., Cohen B. I., Fazzini, E. P., Sarwal, A. N., & Takahashi, M. (1980). Protective effect of plant sterols against chemically induced colon tumors in rats. Cancer Res, 40(2), 403-405.
85. Yasukawa K., Takido M., Matsumoto T., Takeuchi M., and Nakagawa S. (1991). Sterol and triterpene derivatives from plants inhibit the effects of a tumor promoter, and sitosterol and betulinic acid inhibit tumor formation in mouse skin two-stage carcinogenesis. *Oncology*, 48(1), 72-76.

86. Ali H., Dixit S., Alqahtani S. M., Alkahtani S., & Alarifi S., (2015). Isolation and evaluation of anticancer efficacy of stigmasterol in a mouse model of DMBA-induced skin carcinoma. *Drug Des Devel Ther,* 9, 2793-2800.
87. Gomez M. A., Saenz M. T., Garcia M. D., and Fernandez M. A., (1999). Study of the topical anti-inflammatory activity of Achillea ageratum on chronic and acute inflammation models. *Z Naturforsch C,* 54(11), 937-941.
88. Saleem M., Murtaza I., Tarapore R. S., Suh Y., Adhami V. M., Johnson J. J., Siddiqui I. A., Khan N., Asim M., Hafeez B. B., Shekhani M. T., (2009). Lupeol inhibits proliferation of human prostate cancer cells by targeting beta-catenin signaling. *Carcinogenesis,* 30(5), 808-817.
89. Lopez-Lazaro M., Pastor N., Martin-Cordero, C., Navarro, E., Cortes, F., Ayuso, M. J., and Toro M. V., (2003). Anti-tumour activity of Digitalis purpurea L. subsp. heywoodii. *Planta Med,* 69(8), 701-704.
90. Singh, N. K., Singh, V. P., (2014). Anticancer activity of the roots of Ichnocarpus frutescens R. Br. and isolated triterpenes. *Pak J Pharm Sci,* 27(1), 187-191.
91. Tilaoui M., Mouse H. A., Jaafari A., & Zyad A., (2014). Differential effect of artemisinin against cancer cell lines. Nat Prod Bioprospect, 4(3), 189-196.
92. Yoshiaki A., Morio Y., Saori Y., Takashi Y., Daigo W., Masashi H, Sumiko H., Toshihiko H., and Yukihiro G. (2013). Characterization of Phenolic Constituents from Ephedra Herb Extract. *ISSN 1420-3049.*

Appendices

Appendix 1: ^{1}H NMR spectrum of anise pure active V fraction.

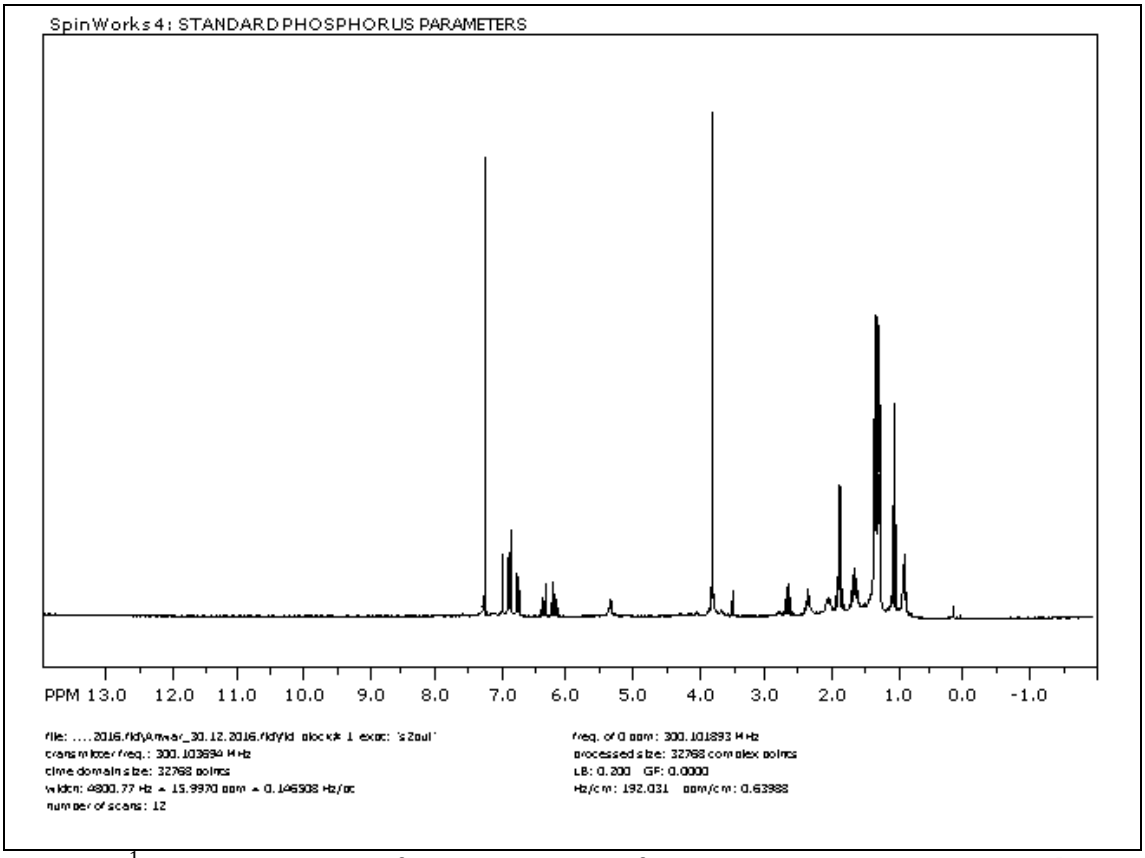


Fig. 40: ^{1}H NMR spectrum of anise pure active fraction V using 300 MHz, sample concentration 10 mg in 2000 µl $CDCl_3$.

Appendix 2: HR-ESi-MS of anise pure active V fraction using electrospray positive mode (+ESi).

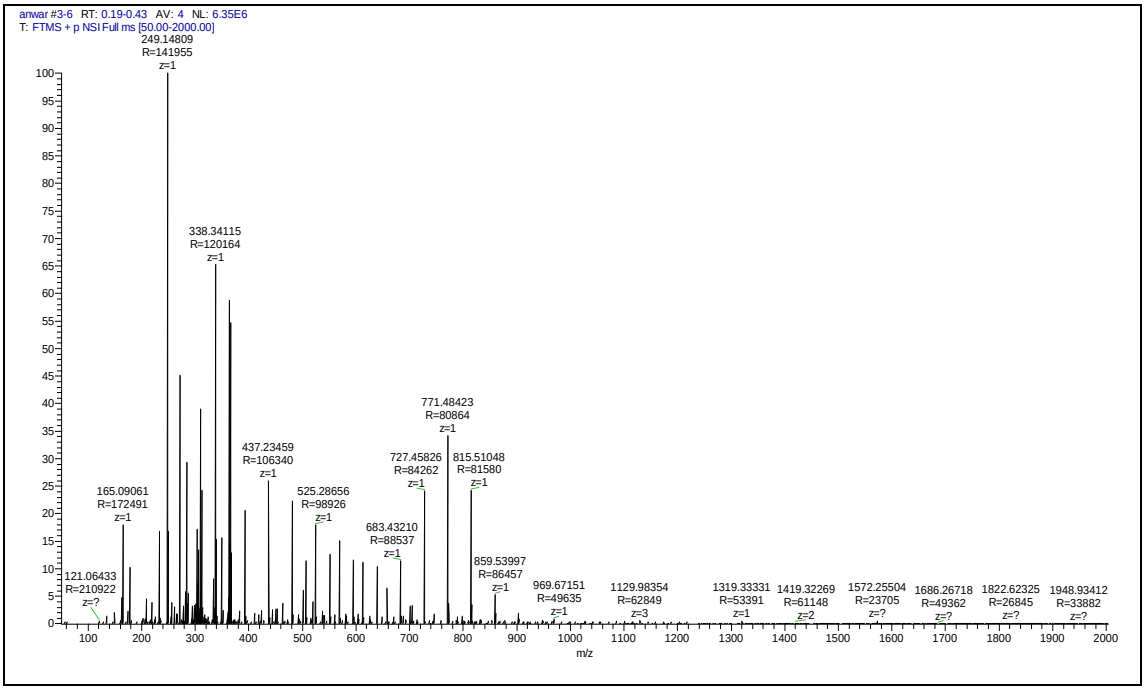


Fig. 41: Typical positive electrospray ionization spectrum of anise pure active V fraction.

Appendix 3: HR-ESi-MS of anise pure active V fraction using the electrospray negative mode (-ESi).

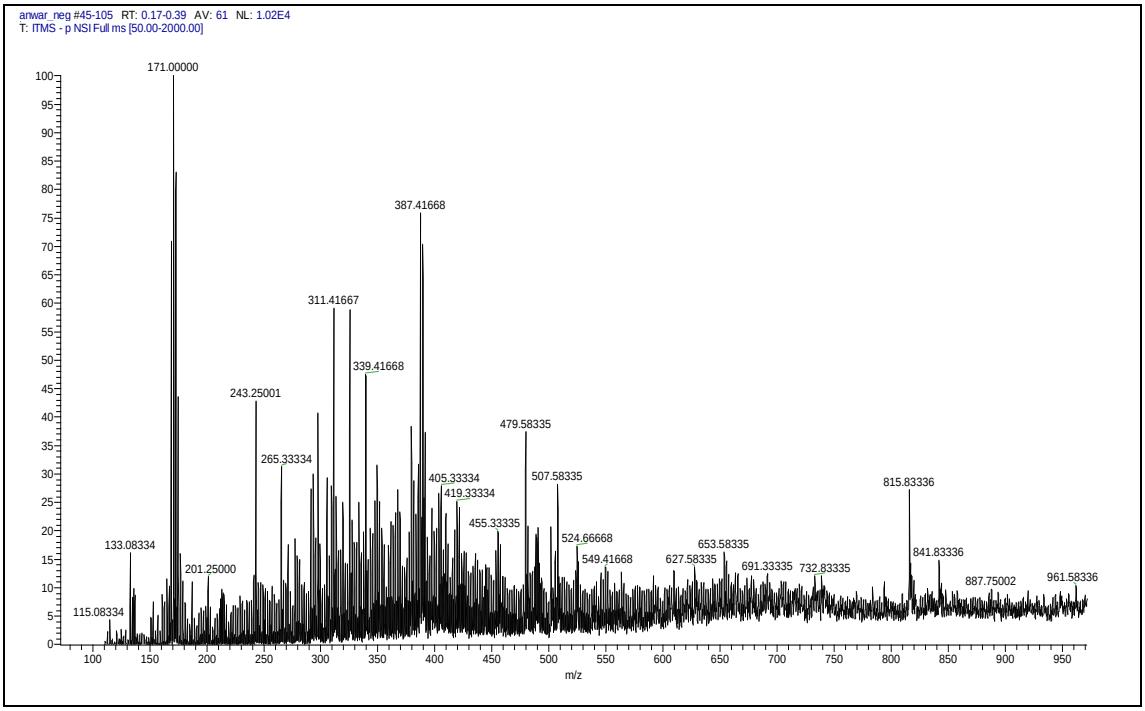


Fig. 42: Typical negative electrospray ionization spectrum of anise pure active V fraction.

مسح المواد الكيميائية الموجودة في النباتات الطبيعية لكل من الحامول الفلسطيني، العكوب الجبلي، اليانسون و العلندى المجنحة وتأثيرها السُمي على الخلايا المخبرية

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اشراف: البرفسور صالح ابو لافي

الملخص

الهدف من هذا البحث هو التقييم المخبري للنشاط الحيوي في تثبيط نمو الخلايا السرطانية من بعض المستخلصات النباتية وبعض مركباتها النقية التي تم تحضيرها بوساطة جهاز (preparative-HPLC) في الحامول الفلسطيني، العكوب الجبلي، اليانسون والعلندى المجنحة. ولقد استخدمت لهذا الغرض تقنيات تحليل الية متقدمة مثل الكروماتوغرافيا السائلة (HPLC-PDA)، وكروماتوغرافيا الغازية ومطياف الكتلة (GC-MS) ، والرنين النووي المغناطيسي لذرة الهيدروجين (^{1}H-NMR)، و(LC-MS) واستخدمت الفحوص البيولوجية لهذه المواد والمستخلصات على الخلايا السرطانية لتحقيق هذا الهدف.

نبات الحامول الفلسطيني مصنف من النباتات البرية الفلسطينية الانتهازية، تم استخدام مستخلصي الميثانول والهكسان لتحديد فعاليته ضد خلايا القولون والمستقيم السرطانية البشرية، النتائج المخبرية تمت بإستخدام اختبار ال(MTT) واختبار ال(LDH)، حيث كانت قيمة التركيز الفعال النصفي (EC_{50}) 4.75 ± 71.55 ميكروغرام\ملليلتر لمستخلص الميثانول، و 3.89 ± 175.21 ميكروغرام\ملليلتر لمستخلص الهكسان، المستخلص الميثانولي اثبت ببذل جهد معتبر في تثبيط نمو خلايا القولون السرطانية (HCT-116)، اما بالنسبة للمواد الفعالة فيه فكانت مادة ال(Sesamin) المشهورة والموجودة في زيت السمسم ولكن كميتها في الحامول الفلسطيني بلغت ثلاثة اضعاف تقريبا مقارنة عما هو موجود بالسمسم، وهذه المادة الفعالة معروفة بقدرتها على تقليل الالتهابات وتقليل الضغط العالي ومحاربة السرطان وكذلك تواجد اثنين من الاستيرولات النباتية (Campesterol) و(Stigmasterol) وبالتالي يمكن ان يكونوا مصدرا رئيسيا فعالاً ضد السرطان.

اما بالنسبة الى نبات العكوب الجبلي فهو يشبه الخُرفيش ويعتبر من الأكلات البرية الشوكية شائعة الاستخدام في المطبخ الفلسطيني. تم التحقق بتأثيرها المضاد للسرطان على خلايا القولون السرطانية البشرية. وجد أن لمستخلصي الميثانول والهكسان فعالية ضد االورم لخلايا (HCT-116) السرطانية بقيمة التركيز الفعال النصفي (EC_{50}) 12 ± 303.3 ميكروغرام\ملليلتر، 18.6 ± 313.3 ميكروغرام\ملليلتر على التوالي، بينما المستخلص المائي كان غير فعال. تم التعرف على هوية حوالي سبعين مركب كيميائي نباتي في مستخلصي الميثانول و الهكسان بواسطة جهاز الكروماتوغرافيا الغازية ومطياف الكتلة GC–MS. ستة من هذه المركبات المفصولة تتمتع بفاعلية قوية ضد السرطان وهي Sitosterol، Stigmasterol، Lupeol، Gitoxigenin، α-

Amyrin و Artemisinin. وكذلك تم جمع ثمان اجزاء منفصلة (fractions I-VIII) من النبات في ثمان قوارير بوساطة (preparative-HPLC) وتم الفحص المخبري لهم. اما الجزء الثامن قليل الذوبان بالماء من النبتة فقد وجد لها الفاعلية الأعلى على خلايا (HCT-116) السرطانية

وبخصوص نبات اليانسون الشهير فلقد وجد له فعالية قوية ضد السرطان على خلايا البروستاتا السرطانية البشرية (PC-3) لذلك تم فصل وجمع ست اجزاء بكميات كبيرة في ست قوارير (fractions from I-VI) باستخدام (preparative-HPLC).وكانت قيم التركيز الفعال النصفي التي تم الحصول عليها كلٍ من الاجزاء II، III، IV و V، بإختبار ال(MTT) هي، 0.10 ± 0.5 مليغرام\ملليتر، 0.11 ± 0.3 مليغرام\ملليتر، 0.12 ± 0.49 مليغرام\ملليتر و 0.10 ± 0.23 مليغرام\ملليتر، على التوالي.

واخيرا فلقد اكتسبت نبتة العلندى المجنحة مؤخرا سمعة كبيرة بعد استخدام مستخلص السيقان في علاج مرض السرطان لمزارع فلسطيني من جنين. ولقد كانت فعالية تثبيط نمو الخلايا السرطانية المخبرية للمستخلص الميثانولي للعلندة (EC_{50}) هي 1.2 ± 25 ميكروغرام\ملليتر باستخدام اختبار ال (MTT) ضد خلايا الكبد السرطانية البشرية HepG2.

ان ما تم اكتشافه من نتائج ايجابية مشجعة في النباتات الفلسطينية (البرية و المزروعة) الأربعة المذكورة سواء من المستخلصات او الاجزاء او المواد النقية الفعالة ليدل على امكانية إجراء المزيد من الابحاث للتوصل الى عقارات جديدة مضادة لأنواع من السرطان ، واهمية دراسة الية عمله وتقييم الفعالية والسُمية في حيوانات التجارب.